Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

In vitro characterization of the antimalarial activity and mode of action of new PDEs and DHODH inhibitors against *Plasmodium falciparum*

Jessy Rosanily Tavares Landim Silva

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Parasitologia Médica.

JANEIRO, 2019

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

In vitro characterization of the antimalarial activity and mode of action of new PDEs and DHODH inhibitors against *Plasmodium falciparum*

Autor: Jessy Rosanily Tavares Landim Silva

Orientadora: Investigadora Doutora Maria de Fátima Carvalho Nogueira

Coorientadora: Investigadora Mestre Lis Tavares Coelho Lobo

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Parasitologia Médica

« SI KA BADU , KA TA BIRADU »

EUGÉNIO TAVARES

Agradecimentos / Acknowledgments

Aos meus queridos pais que sempre me apoiaram incondicionalmente neste caminho, sem nunca duvidar de mim. Muito obrigada por tudo, estou aqui hoje graças a vocês. NTA AMA NHOS TCHEU.

À minha orientadora, investigadora doutora Fátima Nogueira pela confiança, determinação paciência e pela transmissão dos seus conhecimentos. Um grande OBRIGADA por toda a ajuda e dedicação demonstrado a cada momento, pela preocupação e compreensão demonstradas ao longo de toda esta etapa.

À minha coorientadora investigadora Lis Tavares Coelho Lobo, pela orientação, transmissão de conhecimento, dedicação e apoio em todos os momentos. Um grande OBRIGADO por tudo!

Aos meus Tios Delaman e Silvino Silva, e a família pelo amor e suporte incondicional demonstrado sempre.

Aos meus avós, por incutirem em mim essa vontade de querer sempre aprender mais.

À Vera, ao Rafael e ao Helio pelo apoio e companheirismo que demonstraram sempre.

À Cintia, Júlia, Josemar, Mónica, Betty e Luis pelo apoio e suporte nos momentos menos bons, por todos os incentivos que me deram em todos os instantes e por fazerem sempre sentir-me mais forte quando precisei. Mi é dod na nhos sempre.

A todas as pessoas que direta ou indiretamente me ajudaram a levar essa mais uma etapa da minha vida até ao fim.

MUITO OBRIGADA

RESUMO

A malária é uma das doenças parasitárias mais antigas e continua a ser a que mais mortes provoca mundialmente atualmente. Pode ser causada por cinco espécies distintas de protozoários do género *Plasmodium* (*P. falciparum, P. ovale, P. vivax, P.malariae e P. knowlesi*), sendo o *P. falciparum* a espécie mais letal. O parasita é transmitido ao ser humano pela picada do mosquito fêmea do gênero *Anopheles*. aquando da refeição sanguínea. Em 2017, a Organização Mundial da Saúde relatou 219 milhões de casos de malaria em todo o mundo, sendo a grande maioria na região subsaariana do continente africano. A doença pode ser classificada como: malária grave e não grave, afetando principalmente crianças com idade inferior a cinco anos. As resistências aos fármacos antimaláricos em uso, bem como a inexistência de uma vacina eficaz e o difícil controlo vectorial são os principais obstáculos no combate à doença. A disseminação das resistências torna imperativo a investigação e síntese de novos compostos com potencial ação antimalárica, capazes de atuar em diversos estádios do desenvolvimento do parasita, rapidamente.

Neste trabalho, procedeu-se à caracterização da atividade antimalárica e do modo de ação de compostos sintetizados. Compostos sintetizados para serem inhibidores de fosfodiesterases (PDEs) e de dihydroorotate dehydrogenase (DHODH) parasitárias (enzimas importantes para os diferentes estádios de desenvolvimento de *P. falciparum*).

Para a análise da atividade antimalárica, foi avaliado a velocidade de ação dos compostos, os estádios parasitários em que os compostos têm maior atividade, bem como a sua toxicidade nesses estádios. Foram também otimizados dois protocolos de modo a poder, posteriormente, avaliar a ação dos compostos na produção de espécies reativas de oxigénio (ROS) e na alteração do potencial da membrana mitocondrial do parasita.

Avaliando a atividades antimaláricas dos potenciais inibidores de *PF*PDE, um dos compostos demostrou ser de atuação rápida, com atividade nos dois estádios eritrocitários e ter um efeito citocida nos parasitas. O segundo composto com potencial ação contra os *PF*PDEs avaliado, demostrou ser de atuação mais lenta, com uma ação predominante nos parasitas no estádio de anel, mas tendo um efeito citocida. O composto com potencial ação contra os *PF*DHODH demostrou ser de atuação lenta, com uma ação predominante com um efeito citocida nos parasitas no estádio de anel e um efeito citostático nos parasitas no estádio de trofozoítos.

Palavras-chave: malária, *Plasmodium falciparum*, antimaláricos, modo de ação, PDEs, DHODH, ROS, potencial da membrana mitocondrial.

Abstract

Malaria is one of the oldest parasitic diseases and continues to be the one that causes the most deaths worldwide nowadays. It can be caused by five distinct species of *Plasmodium* (*P. falciparum, P. ovale, P. vivax, P.malariae* and *P. knowlesi*), with *P. falciparum* being the most lethal. The parasite is transmitted to humans by the bite of the female mosquito of the genus *Anopheles* during the blood meal. In 2017, WHO reported 219 million malaria cases worldwide, with the vast majority in the sub-Saharan region. The disease can be classified as: severe and non-severe malaria, mainly affecting children under five years old. Resistance to antimalarial drugs in use as well as the lack of a effective vaccine and the difficult vectorial control are the main obstacles in the fight against the disease. The dissemination of resistance makes it imperative to investigate and synthesize new compounds with potential antimalarial action, capable of acting at several stages of parasite development, rapidly.

In this work, the antimalarial activity and the mode-of-action of synthesized compounds were studied. Compounds synthetized to be phosphodiesterase's (PDEs) and dihydroorotate dehydrogenase (DHODH) inhibitors (important enzymes for *Plasmodium falciparum* different stages of development).

For the analysis of the antimalarial activity, the speed of action of the compounds was evaluated, the stage-specific activity and toxicity of the compounds were determinate. Two protocols were also optimized, in order to evaluate, later, the action of the compounds, in the production of reactive oxygen species (ROS) and in the alteration of mitochondrial membrane potential.

Evaluating the antimalarial activities of the potential inhibitors of *PF*PDE, one of the compounds was shown to be fast acting with activity in both erythrocyte stages studied and to have a cytocidal effect on the parasites. The second compound with potential action against the *PF*PDEs evaluated, showed to be of slower action, with a predominant action in the ring-stage parasites, but having a cytocidal effect. The compound with potential action against *PF*DHODH shown to be a slow acting compound, with a predominant action in the ring-stage parasite and to have a cytocidal effect on the ring-stage parasites and a cytostatic effect on trophozoite-stage parasites.

Key words: malaria, *Plasmodium falciparum*, antimalarials, mode of action, PDEs, DHODH, ROS, mitochondrial membrane potential.

Table of contents

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

- **ACTs** – Artemisinin-based combination therapy's
- **AMD** – Amodiaquine
- **ART** – Artemisinin
- **AS** – Artesunate
- **AS/MEF** – Artesunate/Mefloquine
- **ATM** – Artemether
- **ATM/LUM** – Artemether/lumefantrine
- **ATP** – Adenosine triphosphate
- **ATQ** – Atovaquone
- **cAMP** – cyclic Adenosine monophosphate
- **cGMP** – cyclic Guanosine monophosphate
- **CM-H_2DCFDA** – 2',7'dichlorofluorescein
- **CQ** – Chloroquine
- **DAPI** – 4'6'-diamidini-2-phenylindole
- **DDT** – Dichlorodiphenyltrichloroethane
- **DHA -** Dihydroartemisinin
- **DHA/PPQ** – Dihydroartemisinin/piperaquine
- **DHFR** – Dihydrofolate reductase
- **DHODH** – Dihydroorotate Dehydrogenase
- **DHPS** – Dihydropteroate synthase
- **DNA** – Deoxyribonucleic acid
- **DV** – Digestive vacuole
- **EDTA** – Ethylenediamine tetraacetic acid- an anticoagulant
- **HIV** – Human Immunodeficiency Virus
- **IC_{50}** – Half maximal inhibitory concentration
- **IRS** – Indoor Residual Spraying
- **ITNs** – Insecticide-treated bed-nets
- **LUM** – Lumefantrine
- **MEF** – Mefloquine
- **MMV** – Medicines for malaria venture
- **mETC** – mitochondrial electron transport chain
- ***P. falciparum*** – *Plasmodium falciparum*
- ***P. vivax*** – *Plasmodium vivax*
- ***P. ovale*** – *Plasmodium ovale*
- ***P. malariae*** – *Plasmodium malariae*
- ***P. knowlesi*** – *Plasmodium knowlesi*
- **PBS** – Phosphate buffered saline solution
- **PCR** – Polymerase chain reaction
- **PDEs** – Phosphodiesterase's
- ***Pf*dhps** – *Plasmodium falciparum* dihydropteroate synthase
- ***Pf*dhfr** – *Plasmodium falciparum* dihydrofolate reductase

- ***Pf*PDEs** – *Plasmodium falciparum* phosphodiesterase's
- ***Pf*DHODH** – *Plasmodium falciparum* Dihydroorotate Dehydrogenase
- **PPQ** – Piperaquine
- **PQ** – Primaquine
- **QN** – Quinine
- **RBCs** – Red-blood cells
- **RDTs** – rapid diagnostic tests
- **RNA** – Ribonucleic acid
- **ROS** – Reactive oxygen species
- **RPM** – revolutions per minute
- **RPMI** – Roswell Park Memorial Institute medium
- **S/P** – Sulfadoxine/pyrimethamine
- **UV** – Ultraviolet
- **WHO** – World Health Organization
- **3D7** – *P. falciparum* strain sensitive to chloroquine and mefloquine
- **3D7HT-GFP** – A genetic recombinant *P. falciparum* strain constructed by integration of green fluorescence protein

I-INTRODUCTION

I.1. MALARIA

Malaria is one of the oldest diseases known to affect human kind. The characteristic periodic fever of the disease is described in antique Chinese literature dating back to 2700 BC. Hippocrates (5^{th} century BC) was the first to describe the disease. However, only in the 19th century, the causative agent of the disease was identified. In 1897, Ronald Ross discovered *Plasmodium falciparum* oocysts in an anopheline mosquito fed on patients suffering from malaria, and later was able to observe all the stages of the parasite as also described by Laveran in mosquitos fed on infected malaria birds (Knell, 1991).

Malaria is distinguished in two categories by the World Health Organization (WHO), uncomplicated malaria and severe malaria. The first symptoms of the disease are identical to a common viral illness and can appear up to fifteen days after the contact with the vector. It provokes headache, fatigue, abdominal discomfort, muscle and joint aches, fever usually followed by chills and perspiration, anorexia, and vomiting. In young children, it usually also causes lethargy, loss of appetite and cough (White *et al.*, 2014). It is considered uncomplicated malaria when patients present the symptoms presented above and a positive parasitological test diagnostic (microscopy or rapid diagnostic test). If not treated correctly in the first twenty-four hours, it can develop into severe malaria and to death. It is considered severe malaria when the patient present one of the following symptoms, with no other identified alternative cause and in the presence of *P. falciparum* asexual parasitaemia according to WHO. The symptoms observed in severe malaria can be cerebral coma, severe anaemia, hypoglycaemia, metabolic acidosis, acute renal failure and acute pulmonary oedema. Severe malaria is fatal in most of the cases (WHO, 2015). The treatment is focused on eliminating all the parasites from the body of the patient, preventing the passage to severe malaria, avoiding malaria relapse and recrudescence, interrupt the transmission of the parasite and to prevent the emergence and spread of resistance to antimalarial drugs. WHO recommends different treatments according to if it is considered uncomplicated malaria, severe malaria or if it is patients with uncomplicated malaria in special risk groups (pregnant women, infant with less than 5 Kg body weight, patients co-infected with HIV, non-immune travellers, patients with uncomplicated hyperparasitaemia) (WHO, 2015).

I.2. MALARIA: GEOGRAPHICAL DISTRIBUTION AND GLOBAL EPIDEMIOLOGY

Malaria remains one of the deadliest vectors born-diseases in low-income countries. Nearly half of the world's population was at risk of contracting malaria in 2017 according to the WHO. Malaria is considered endemic in the tropical and subtropical regions (Figure I.1) and affect mainly sub-Saharan where 93% deaths due malaria occurs. In other parts of the world it causes considerable morbidity, particularly in rural areas of the countries of the South-East Asia region and the eastern Mediterranean region. The WHO has estimated that the incidence rate of malaria and malaria deaths rate has decreased by 37% and 60% globally between 2000 and 2015 but despite these reductions, a substantial increase in malaria cases has been estimated since 2015. WHO reported an increase of five million clinical cases in 2016 compared to 2015 and a total of 445 000 deaths worldwide(WHO, 2015b).

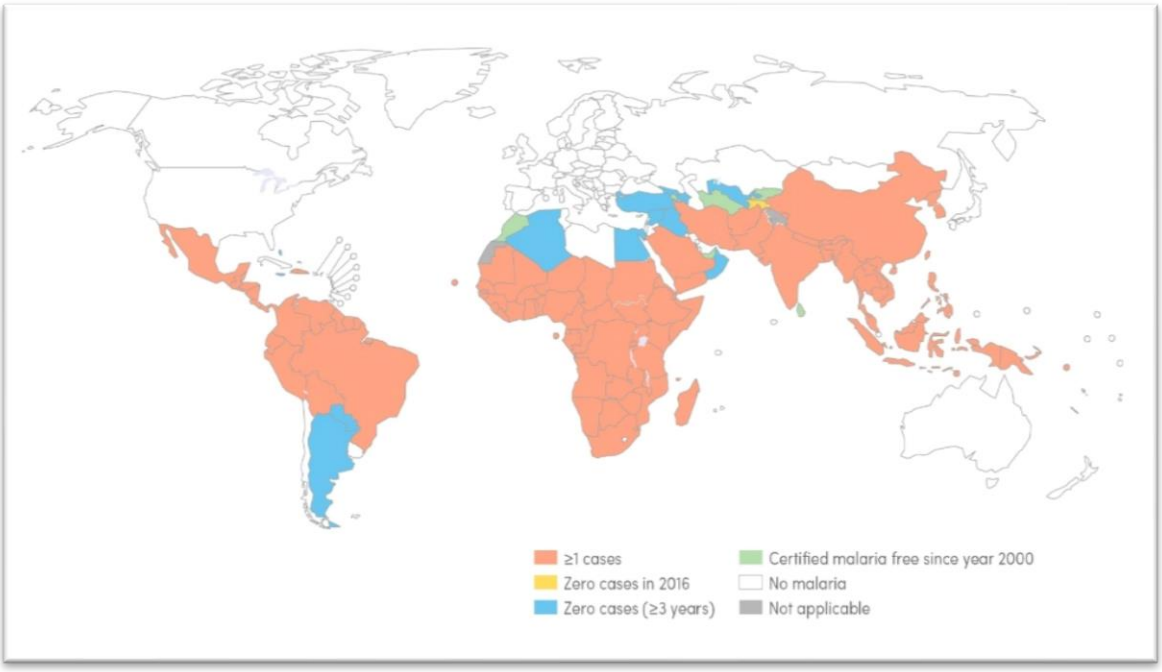

Figure I.1. Distribution of indigenous cases of Malaria in 2016. Adapted from (WHO, 2017).

Plasmodium spp. are transmitted to humans by female *Anopheles* mosquitos' bites during the blood meal that can take place indoors or outdoors. The disease can be caused by five different species of the genus *Plasmodium*: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi,* with *P. falciparum* being the deadliest to humans. Every person can be infected by the protozoan, but some groups in the population such as children under five years old, pregnant women and immunocompromised peoples are more likely to be infected and to be more seriously ill (Ashley *et al.*, 2006).

I.3. LIFE CYCLE OF THE PARASITE

The parasite has a complex life cycle (Figure I.2), in which it needs two hosts to be able to complete; some hematophagous female of the *Anopheles* mosquitos are the definitive hosts in which occurs the exogenous sexual phase, called sporogony phase and some vertebrates are the intermediate hosts in which occurs the endogenous asexual phase called schizogony phase (Antinori *et al.*, 2012). The sporogony phase take place after a blood meal in an infected vertebrate in which the mosquitos ingest gametocytes (sexual stage - male and female) that fuse in the midgut to form zygotes that develops into oocysts. After maturation, the oocysts grow into sporozoites and migrates to the salivary glands of the mosquitos where it can be transferred to a new vertebrate host during the blood meal. In the vertebrates, sporozoites migrates to the liver where they develop into schizonts and the schizonts divide by asexual reproduction generating merozoites. This phase is called preerythrocytic phase. It can last about one to two weeks and can be asymptomatic. After rupture of the hepatocytes, the merozoites enter the bloodstream and infects erythrocytes. In the red blood cells, some merozoites develops, into gametocytes and some develops into young trophozoites called "rings", which mature in trophozoites and then into schizonts, consuming haemoglobin. Gametocytes, the stage that transmit the infection, has four stages of developments in which in the fourth occurs the differentiation into male and female, are "banana" shaped and can take seven to ten days to develops in *P. falciparum*. Schizonts after maturing, rupture the cells releasing more merozoites into the bloodstream thus restarting the cycle. The rupture of the blood cells provokes the symptoms characteristic of malaria, the fever. This phase is called erythrocyte phase and can last until lysis of the erythrocytes if left untreated. In the infection by *P. falciparum*

all the schizonts mature into merozoites. In the infection by *P. vivax* and *P. ovale*, the merozoites may develop latent hepatic forms, the hypnozoites, and begin a new erythrocyte cycle months or years later (Cowman *et al.*, 2016).

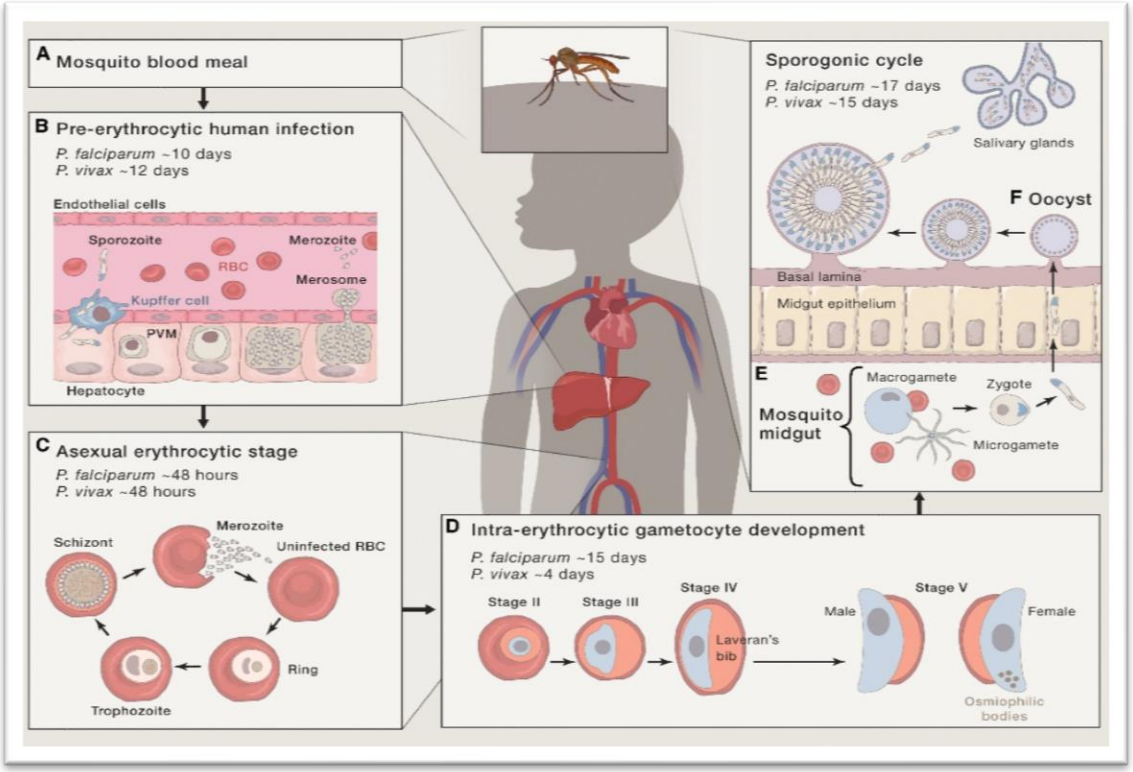

Figure I.2. Life cycle of *Plasmodium* spp. Adapted from (Cowman et al., 2016).

I.4. MALARIA CONTROL

Presently WHO's strategy to control and eliminate malaria, is based on: vector control; the development of an effective vaccine; chemotherapy; and chemoprophylaxis. The vector control mainly aims to reduce the vectorial capacity of the vector below the critical threshold necessary for the transmission of the parasite. This purpose is achieved by decreasing the number of vectors on the transmission phase and by limiting the contact frequency between the vector and the human population. The main vector control methods used nowadays are based on ecological, biological, chemical and genetic control. Briefly, the ecological control can be directed to the larval or/and the adult form of the

mosquitos by improving health infrastructures and managing aquatic habitats potentially favourable to mosquito breeding. Biological control is based on the use of synthesized biological products that can change the development and/or behaviour of the vector or by introducing natural enemies in the ecosystem. Chemical control is achieved by applying outdoor and/or indoor residual spraying (IRS), insect repellents and using insecticide-treated bed-nets (ITNs). Genetic control of the vector, in turn, is based on the principle of causing or introducing in the vector genetic alterations that are disadvantageous to the vector or to the pathogen it carries, and which can lead to its elimination (Tizifa *et al.*, 2018).

Due to the complexity of malaria parasites biology, the development of an effective vaccine against the disease hasn't been successful so far. There is no available malaria vaccine in the commerce currently, but more than 20 vaccine constructs are currently being evaluated in clinical trials or are undergoing preclinical development and recent progress has been made with the completion of a Phase 3 trial of the RTS, S/AS01 candidate vaccine (WHO, 2017).

Since 2010, for the chemotherapy in the case of uncomplicated malaria, WHO recommends artemisinin-based combination therapy (ACTs) when the disease is caused by the *P. falciparum* parasite. When the disease is caused by *P. vivax*, *P. ovale*, *P. malariae* or *P. knowlesi* the treatment is based on chloroquine-based treatment, primaquine or ACTs when it is a zone with chloroquine-resistant parasites. The treatment is recommended to be done during three successive days to ensure the adequate efficacy and minimize the risk of the drug resistance due to incomplete treatment. When the disease in considered severe malaria, WHO recommend treating patients with intravenous or intramuscular artesunate for minimum 24h until they can tolerate oral medication and once it is possible, a three consecutive days ACTs treatment. For patients in special risk groups, WHO preconise different approaches as appropriate. For women in the first trimester of pregnancy, for example, a seven-day treatment with clindamycin in addition to quinine, is recommended to avoid adverse effects on the mother or the foetus that may be cause by others antimalarials.

Chemoprophylaxis is based on the use of complete treatment as prophylaxis and is recommended when traveling to affected areas, during the potential exposition to malaria and up to four weeks after the exposure (Walker, Nadjm and Whitty, 2018). WHO presently recommended three preventive therapies against the disease: Intermittent preventive malaria treatment in pregnancy (IPTp) and in infant (IPTi) with sulfadoxine-pyrimethamine; and seasonal malaria chemoprevention for children aged 3-59 month to avoid the disease (WHO, 2017).

Malaria is a preventable and curable disease if diagnosed in time. The main diagnosis test used is the thick and thin blood film Giemsa stained microscopy test. Useful for detecting blood-stage parasite, the thick blood film test, a sensitive test, indicates the presence or the absence of parasites in the blood, meanwhile the thin blood film test, a more specific test, indicate the specie and abundance of the parasite in the blood. Immunochromatographic rapid diagnostic tests (RDTs), also a sensitive test, detect antigens or parasite-specific enzymes. It is also widely used to detect the presence or absence of the parasite in the bloodstream, principally in remote areas with limited access to good microscopy services, (WHO, 2017) or as an additional test in non-endemic areas where few cases are observed annually (Walker, Nadjm and Whitty, 2018). The Polymerase chain reaction (PCR), a very sensitive test, is used to detect low-intensity malaria infections, but due to its elevated cost, low availability and the amount of time needed to obtain the results, the WHO only recommended using it for epidemiological research and sub-microscopic infection mapping.

I.5. THE PARASITE

The protozoan parasites belonging to the *Plasmodium* genus, is the causative agent of the disease. It's a unicellular *Eukaryota*, belonging to the *Apicomplexa* phylum, to the *Aconoidasida* class, to the *Haemosporida* order and to the *Plasmodiidae* family (Antinori *et al.*, 2012). *Plasmodium spp* (like other apicomplexans parasites) has several structures. The rhoptries containing parasite proteins that help in the invasion and modification of the host once the parasite is inside. The micronemes that contains parasite proteins indispensable for mobility, identification and attachment to host cells. Spread all over the

parasite, dense granules containing parasite proteins involved in the modification of the membrane that separates the parasite from the host (Morrissette and Sibley, 2002). The parasite is also composed by a single large mitochondrion and the apicoplast, two organelles of endosymbiotic origin, who are indispensable for the parasite's metabolism. The mitochondrion is responsible for the production and transport of energy in form of Adenosine triphosphate (ATP) by means of the electron transport chain. The apicoplast, in turn, is involved in the synthesis of different metabolic mechanism such as protein, DNA and isoprenoid synthesis. The digestive vacuole (DV) is involved on the haem polymerization, the haemoglobin hydrolysis and free-radicals generation (Fidock *et al.*, 2004).

I.6. ANTIMALARIAL DRUGS

I.6.1. CURRENT ANTIMALARIAL DRUGS

Antimalarial drugs primarily aim at eliminating blood stage parasites as quickly as possible, so as to prevent the disease to progress to more severe forms and even death. In terms of public health, it is intended to reduce the transmission of infection among the population and to prevent the emergence and dispersion of resistance to antimalarials (WHO, 2016).

Since 2010, WHO recommends only administering antimalarials following confirmatory diagnosis by microscopy or rapid diagnostic tests (WHO, 2015a). Since 2001, WHO has recommended that artemisinin-based combination therapy (ACT), by 2010 most malaria endemic countries had shifted their national treatments policies to ACTs (WHO, 2010). ACTs consist of combining an artemisinin derivative (ARTs) with a partner drug, with a longer half-life, different pharmacophore and mechanism of action (WHO, 2015). In ACTs, the artemisinin derivative (being a fast-acting drug) plays a role in drastically reducing the number of parasites in the first 3 days of treatment. While the partner drug (slower acting) eliminates the residual parasites (WHO, 2015). Some of the other advantages that lead to the use of ACT are: to rapidly reduce parasitaemia, rapid resolution of clinical symptoms, action against multidrug resistant *P. falciparum* and have

activity against gametocytes, allowing a decrease in transmission (WHO, 2015). Drug regimens for treatment of the two most prevalent malaria parasites, *P. falciparum* and *P. vivax,* are different. Chloroquine (CQ) plus primaquine (PQ) remain the first-line treatment for radical cure of *P. vivax* malaria in most malarious regions (Walker, Nadjm and Whitty, 2018). Available antimalarial drugs can be divided into multiple classes (Table I.1).

The first important antimalarial drug discovered in history was the alkaloid quinine, from the "Peruvian fever tree" Peruvian bark *Cinchona* named by Linnaeus in 1749 (Knell, 1991). It was brought to Europe from South America in the 17th century, because of its known medical values for curing and treating fevers. Quinine and its derivatives are known to target blood stages of the parasite, particularly trophozoites and early stage gametocytes, causing clumping of hemozoin and producing nuclear and cytoplasmic degeneration (Knell, 1991;Haldar, Bhattacharjee and Safeukui, 2018). Quinine (QN) is an arylamino alcohol that is the oldest antimalarial drug used as cinchona bark since the 1600s and its pure since 1820 (Rosenthal, 2001). It produces severe side effects, and its use is practically limited to the treatment of severe malaria.

Important malaria-related drugs are mefloquine (MEF) and lumefantrine (LUM), components of the ACTs artesunate/mefloquine (AS/ MEF) and artemether/lumefantrine (ATM/ LUM).

During the Word War II, an alternative to QN was developed since it was no longer available to the US troops. They were able to produce CQ, belonging to a new class of antimalarials; the 4-aminoquinolines. CQ targets the polymerization of free haem into a crystal called hemozoin, within the food vacuole of trophozoites and schizonts. The 4-aminoquinoline, CQ, was the mainstay for the treatment of uncomplicated malaria for decades. Due to its low price and negligible side effects, CQ became (together with the insecticide DDT), the mainstay of malaria control worldwide. A decade after its massive use, the first signs of resistance emerged and by the 1980s CQ-resistant parasites were widely spread in the malarious regions (Butler, Khan and Ferguson, 2010; Haldar, Bhattacharjee and Safeukui, 2018). Currently drug resistance to CQ has spread to nearly

al malarious regions, render it inappropriate for the treatment of *P. falciparum* malaria. In Indonesia, the emergence of *P vivax* resistance to CQ prompted a policy change to ACTs also (Baird, 2011).

Amodiaquine (AMQ) probably has the same resistance mechanisms as CQ, but because it has higher potency it is still effective against many CQ-resistant parasites, and it is a component of the recommended ACTs (Folarin *et al.*, 2011).

The more recently introduced piperaquine (PPQ), was widely used to treat malaria in China for decades, now is declining because of increasing drug resistance (Davis *et al.*, 2005; Ashley and Phyo, 2018). It is currently, a component of the ACT, dihydroartemisinin/ piperaquine (DHA/ PPQ) (WHO, 2015)

The 8-aminoquinoline primaquine (PQ), even thought, it has some activity against blood stage parasites, it is mostly used to eliminate parasite liver stages (and dormant forms - hypnozoites of *P. vivax* and *P. ovale*) (Ashley and Phyo, 2018). PQ is also active against gametocytes, reducing transmission of parasites to mosquito vectors (Ashley and Phyo, 2018). The WHO recommends the use of a single dose of PQ in combination with ACTs for *P. falciparum* malaria treatment in order to decrease transmission (WHO, 2015a).
Antifolates drugs target parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) (Ashley *et al.*, 2006). Sulfadoxine/pyrimethamine (SP) has the advantage of being single-dose therapy, but its efficacy is currently seriously limited by disseminated drug resistance (Barnes *et al.*, 2008). SP, antifolate drugs, began to be used in 1948 and 1967 respectively, targeting liver stage parasites and blood stage parasites, but rapidly presented resistance. SP inhibit the P. *falciparum* enzymes dihydropteroate synthase (*Pf*dhps) and dihydrofolate reductase (*Pf*dhfr), which function in the folate pathway (Haldar, Bhattacharjee and Safeukui, 2018).

The naphthoquinone atovaquone (ATQ), introduced in later in 1996, acts against the mitochondrial cytochrome bc_1 complex (Van Voorhis *et al.*, 2016). Combined with the DHFR inhibitor proguanil the, commercially available Malarone (although expensive therapy), is largely recommended for as chemoprophylaxis to travelers (Ashley *et al.*,

2006). ATQ combined to proguanil dissipate mitochondrial membrane potential leading to the release of cytochrome C and to the apoptosis of the parasite (Haldar, Bhattacharjee and Safeukui, 2018). It is noteworthy that the synergy of this combination appears to be independent of inhibition of folate synthesis (Cui *et al.*, 2015).

Antibiotics such as doxycycline, clindamycin and tetracycline, are also used as antimalarials, because of their action against the protein synthesis machinery of the apicoplast (Dahl and Rosenthal, 2008).

Currently, artemisinin derivatives (ARTs) are the most significant group of antimalarials it was developed from a natural product, extracted from the plant *Artemisia annua*, the active compound was isolated in 1971 in China (Cui and Su, 2009). Artemisinin (ART) is a potent antimalarial. Artemisinin-based combination therapy (ACTs) are currently the most effective and the mainstay of recommended treatment since 2006 by WHO to treat uncomplicated *P. falciparum* malaria. The ARTs artesunate (AS), artemether (ATM) and dihydroartemisinin (DHA) are used as components of the ACTs (WHO, 2015a). ARTs are highly effective against malaria, but have a very short half live, so the combination with a longer-acting partner drug allows short treatments (3-day) that also protects against the selection of resistant parasites (Nosten and White, 2007). AS is also used for the treatment of severe *P. falciparum* malaria, with documented survival advantages compared with QN (Dondorp *et al.*, 2005, 2010; WHO, 2015a).

Five combinations of ACTs are preconized by WHO to use against malaria. Artemether + Lumefantrine; Artesunate + Amodiaquine; Artesunate + Mefloquine; Dihydroartemisinin + Piperaquine, and Artesunate + Sulfadoxine-Pyrimethamine (SP) (WHO, 2017).

Table I.2. Currently used antimalarial drugs and respective targeted parasite stage (adapted from Haldar, Bhattacharjee and Safeukui, 2018)

Chemical Class	Common Name	Target
Sesquiterpene lactone Endoperoxides	Artemisinin	All parasite stages
	Artesunate	
	Artemether	
	Dihydroartemisinin	
4-Aminoquinolines	Chloroquine	Blood stages (trophozoites and schizonts)
	Amodiaquine	
	Piperaquine	
Amino alcohols	Quinine	Blood stages (trophozoites and stage I to III gametocytes)
	Mefloquine	Blood stages (trophozoites and schizonts)
	Lumefantrine	
	Halofantrine	
8-Aminoquinoline	Primaquine	Blood (gametocyte) and liver (schizont) forms
Antifolates	Pyrimethamine	Blood and liver schizont and mosquito stage (oocyst)
	Sulfadoxine	Blood and liver schizont
	Proguanil	Blood stages (schizont and gametocyte) and liver schizont
Naphthoquinone	Atovaquone	Blood stages (schizont and gametocyte) and liver schizont
Antibiotics	Clindamycin	Blood stages
	Doxycycline	
	Tetracycline	

I.6.2. ANTIMALARIALS DRUG RESISTANCE

Drug resistance in malaria is almost exclusively related to *P. falciparum*, and will be the focus of our work, from hereon.

The emergence of CQ resistant malaria was first identified in Southeast Asia and South America around 1960. Subsequently, CQ-resistant parasites spread of Africa increasing up to 3-fold malaria-related deaths, in the 1980s (Petersen, Eastman and Lanzer, 2011). An alternative to CQ was introduced SP however, about a year after implementation SP drug-resistance was identified (Wernsdorfer and Payne, 1991). Parasite resistance to SP spread quickly throughout Southeast Asia, and later to Africa (1990s decade) (Hurwitz, Johnson and Campbell, 1981; Verdrager, 1986), since then has rapidly spread throughout Africa as well (Arrow *et al.*, 2004).

Several other antimalarial drugs have since, been introduced as treatment regimens against malaria, including mefloquine, amodiaquine and quinine (Petersen, Eastman and Lanzer, 2011). The usage of these replacement drugs in monotherapies equally resulted in the selection of resistant parasites, in vast malarious regions

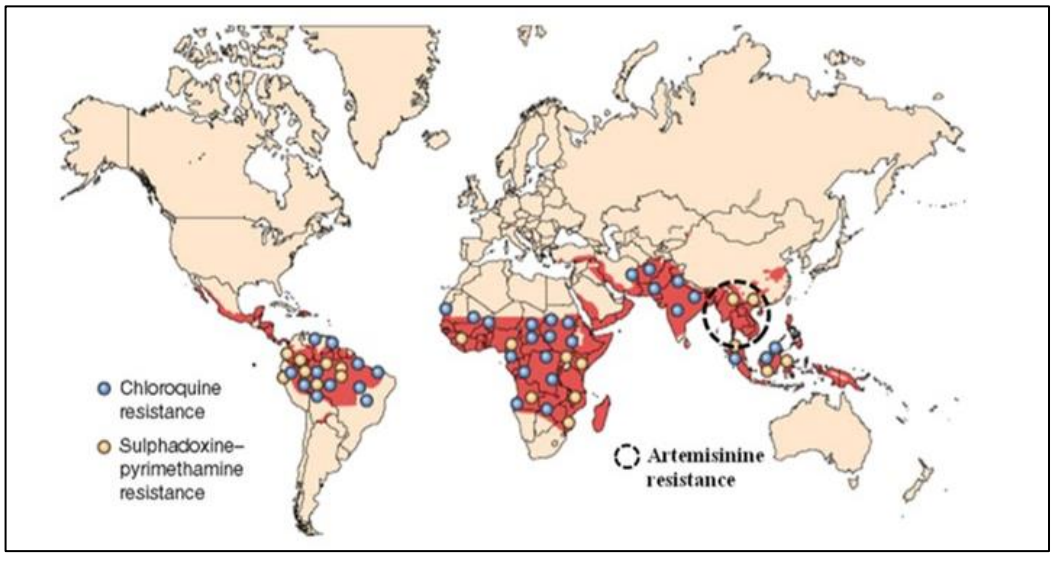

Figure I.3. Geographic distribution of antimalarial drug resistant of *Plasmodium falciparum*, adapted from (Krungkrai and Krungkrai, 2016). Resistance to chloroquine and sulphadoxine-pyrimethamine are represented in blue and yellow circles, respectively. Resistance to artemisinin across the Great Mekong region in 2014, is shown in black dash area.

Mefloquine was introduced as a treatment drug around 1975 (Trenholme *et al.*, 1975), and clinical evidence of parasites resistant to mefloquine began to appear in Southeast Asia around the time of its general availability in 1985 (Hoffman *et al.*, 1985).

The currently used treatment for malaria the ACTs, combine ART derivative with a partner drug of a distinct chemical class (Table I.1) (WHO, 2015). ACTs compensate for the poor pharmacokinetic properties of the ARTs and increase treatment efficacy, while reducing the emergence of drug-resistant parasites (Petersen, Eastman and Lanzer, 2011). Unfortunately, in 2008 reports observed the emergence of ART resistant parasites in Southeast Asia (Petersen, Eastman and Lanzer, 2011). Presently, ART drug resistance is disseminated across the Great Mekong region (Figure I.3) (Blasco, Leroy and Fidock, 2017). The concomitant resistance to ACT partner drugs, resulted in high failure rates to certain ACTs, such as dihydroartemisinin + piperaquine (Leang *et al.*, 2013, 2015; Lon *et al.*, 2014; Duru *et al.*, 2015; Spring *et al.*, 2015; Amaratunga *et al.*, 2016) or artésunate + méfloquine (Carrara *et al.*, 2013). In view of this and before *P. falciparum* malaria becomes untreatable, new antimalarial treatments are urgently needed.

I.6.3 NEW ANTIMALARIALS KILLING PROFILE

The different life cycle stages of *P. falciparum* provide several different targets for antimalarials, but to date, all drugs in clinical use act mainly against the intraerythrocytic development stages (figure I.4.).

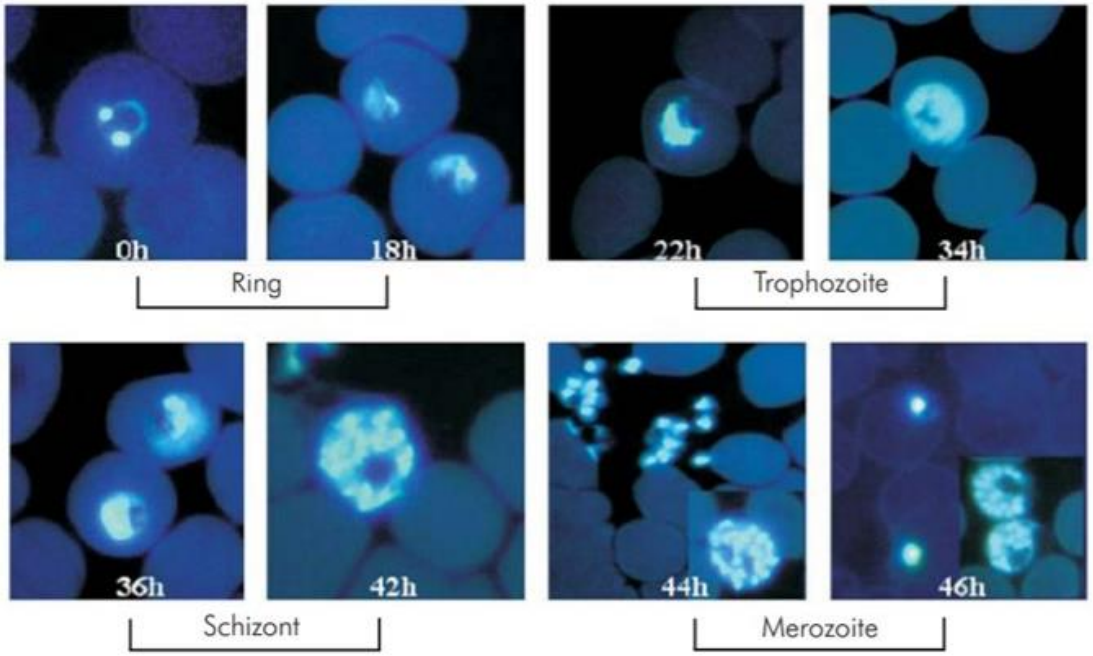

Figure I.4. *Plasmodium falciparum* intraerythrocytic life cycle: stages of development, adapted from (Nogueira and Rosário, 2010). *In vitro* sorbitol synchronized culture parasites stained with DAPI (4',6'-diamidini-2-phenylindole) and photographed under UV light in a fluorescence microscope (1,000 x).

The most widely used drugs, act on the food vacuole and hemozoin formation of rings and trophozoites blood-stages (Zarchin, Krugliak and Ginsburg, 1986; Loria *et al.*, 1999; Famin and Ginsburg, 2002; KRISHNA, UHLEMANN and HAYNES, 2004) or on enzymes in the trophozoite folic acid biosynthesis pathway (Ferone, Burchall and Hitchings, 1969; Yuthavong *et al.*, 2006; Raphemot *et al.*, 2016). Drugs that have been used clinically, have one of these two modes of action and include CQ, AMQ, QN, SP, ARTs (mostly artemether and artesunate) and LUM. Unfortunately, drug resistance has rendered many of these compounds ineffective, and there are reports of ARTs resistant parasites, across the Southeast Asia (Dondorp *et al.*, 2009, 2017; Noedl, Socheat and Satimai, 2009) In fact, the delayed parasite clearance in response to ACTs has been observed clinically and is recognised as the phenotype for ART-resistant parasites (White *et al.*, 2015; Dondorp *et al.*, 2017).

ARTs are fast acting drugs with marked parasiticidal effect, quickly relieving malarial symptoms and minimizing the chance for resistant parasite selection (Burrows *et al.*,

2013). Replacement of ACTs requires the identification of new drugs with rapid parasite killing kinetics including stage specificity activity.

In vitro parasite growth inhibition assays expose parasites to a compound during a period of time and at the end of incubation viability is assessed by labelling nucleic acids or measuring enzymatic activity (Machado *et al.*, 2016). However, these approaches do not directly measure the speed of parasite killing, and the reliability of such tests depends on the drug mechanism of action (Wein *et al.*, 2010; Sanz *et al.*, 2012). A number of different approaches have been proposed for the *in vitro* identification of rapidly parasiticidal anti-malarial drugs have been proposed (reviewed in Sinha *et al.*, 2017).

The development of resistance to antimalarials drugs over the years, the lack of new antimalarials drugs, the need of a fast acting, single dose drug with minimal toxicity and at a low price led to the active research for new drug with new mechanisms of action for the elimination of malaria. Following this purpose, over 6 million compounds against asexual-stage P. *falciparum* have been screened since 2007. This initiative was achieved by pharmaceutical companies (GlaxoSmithKline and Novartis) and by academic centres (St. Jude, Memphis and Eskitis, Australia). The screening has led to the creation of a library of compounds called the "Malaria Box", which is freely distributed to researchers for further studies about different mechanism of action and antimalarial profile (Van Voorhis *et al.*, 2016).

For this thesis, compounds of the "Malaria Box" provided by Medicines for Malaria Venture (MMV), a Swiss non-profit organization that is committed in the development of new antimalarial drugs were used. The 2,3-dihydro-1H-pyrrolo[3.4-b]quinoline-9(4H)-one (named Pyrroloquinolones), known to have a dual-stage activity against malaria parasite is a promising phosphodiesterase 5 (PDE5) (Zheng *et al.*, 2018) and *Plasmodium falciparum* Dihydroorotate Dehydrogenase (*Pf*DHODH) inhibitor. Since there are only few investigations about their antimalarial profile and mode of action, our laboratory in association with PhD Professor Rui Moreira's group decided to study them.

The PDEs represent an important family of enzymes that is essential for the degradation of the cyclic nucleotide in the second messenger signal through hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and

blocking their cellular signals. Presently, PDEs is constituted by 11 gene-related families of isozymes (PDE1 to PDE 11) (Keravis and Lugnier, 2012). High cytoplasmic levels of secondary messengers (cAMP, cGMP) allow the activation of proteins essential for the survival of *P. falciparum* (kinase A and G proteins responsible for the translation of monophosphate cell signals), specifically in gametogenesis and cell invasion (Beraldo *et al.,* 2005). Phosphodiesterase control the production of these messengers, reducing their cellular accumulation (Ramdani *et al.,* 2015). The *P. falciparum* genome encodes four highly similar *Pf*PDEs, of which only two, *Pf*PDEα and *Pf*PDEβ, is related to be expressed in asexual blood stages (Baker *et al.,* 2017a). By using phosphodiesterase inhibitors, the exponential increase of these cyclic nucleotides leads to successive events that inhibits the occurrence of a normal developmental cycle by *Plasmodium falciparum,* principally in the stages of erythrocyte invasion and gametogenesis

Dihydroorotate dehydrogenase (DHODH) was already known to be a promising target for the development of new antimalarial drugs but has been confirmed using evolution-based methods. DHODH is an enzyme localized in the mitochondrial electron transport chain (mETC), which provides oxidized ubiquinone as an electron acceptor for DHODH to synthesize the *de novo* pyrimidine (Luth *et al.,* 2018). Pyrimidines are essential metabolites that are precursors of DNA and RNA biosynthesis. Cells acquire pyrimidines by *de novo* synthesis from ammonia (derived from L-glu), bicarbonate and L-asp, or by recovering preformed bases of pyrimidine (uracil, cytosine and thymine) or nucleosides (uridine, thymidine and cytidine). The parasite cannot save pyrimidines from its host, so the mETC activity is essential for cell growth (Phillips and Rathod, 2010). The use of *Pf*DHODH as a drug target will lead to the collapse of the cell and to the death of the parasite.

II-OBJECTIVES

II-OBJECTIVES

Despite all the advances in research in the fight against malaria, the disease remains one of the deadliest in the world. Characterization of potential antimalarials mode of action, and the search for new and different parasite targets is a powerful tool to be prepared for potential ACT spread of resistance, since there are no other new known antimalarial available.

In this context, this thesis aimed to test three compounds, synthetized by PhD Professor Rui Moreira's group, belonging to the pyrroloquinolones family, known to be potential phosphodiesterase (PDEs) and dihydroorotate dehydrogenase (DHODH) inhibitors against *P. falciparum*.

General objectives

In vitro characterization of the antimalarial activity and mode of action of new PDEs and DHODH inhibitors against *P. falciparum*.

Specific objectives

1 - Evaluate the speed-of-action of the compounds.

2 – Determine the stage-specific activity of the compounds.

3 – Optimization of the protocols of reactive oxygen species and mitochondrial membrane potential.

III-MATERIALS AND METHODS

III.1. REAGENTS

III.1.1. COMPLETE MEDIUM SOLUTION FOR *PLASMODIUM* CULTURE

To prepare one litre of complete medium solution (RPMIc), 10,44g of RPMI 1640 (Gibco), 5,95g of HEPES (sigma) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5g of AlbuMAX II (Invitrogen) and 0,1g of Hypoxanthine (Sigma) were dissolved in Milli-q water. After homogenizing the solvents for 30 minutes, 2g of sodium bicarbonate (Sigma) was added to have a neutral pH of 7,4 like in the human blood. For the storage, the medium was filtered, with a 0.22 µm pore into a sterile container and maintained at 4ºC until its use.

III.1.2. GIEMSA DYE SOLUTION

The Giemsa dye solution at 20% was prepared adding 20 mL of Giemsa (VWR) concentrate in 80 mL of buffered water (Sigma). The solution was then filtered using filter paper and stored at 4ºC.

III.1.3. D-SORBITOL SOLUTION

D-Sorbitol solution at 5% was obtained by adding 50g of D-Sorbitol (VWR) to one litre of distilled water. The solution was filtered using a filter with a 0,22 µm pore into a sterile container and kept at 4ºC.

III.1.4. PHOSPHATE BUFFERED SALINE SOLUTION (PBS)

For a 400 mL PBS solution, 2 PBS effervescent tablets (VWR) were dissolved in 400 mL Milli-q water. The solution was then autoclaved to ensure its sterility and maintained at 4ºC.

III.1.5. CYTOMETER CLEANING REAGENT

To clean the cytometer, a sodium azide solution were prepared. For 100 ml of Milli-Q water, 10g of sodium azide in powder were added.

III.1.6. COMPOUNDS, DYES AND DRUGS

- MS 092
- MS 105
- GS 082
- CQ, Chloroquine
- ATQ, Atovaquone
- CM-H_2DCFDA (Invitrogen^{TM})
- SYTO^{TM}61 (Invitrogen^{TM})
- Rhodamine 123 (Sigma-Aldrich)

The compounds to be tested were synthetized and provided by the colleagues of the "Faculdade de Farmácia da Universidade de Lisboa" advised by the PhD Professor Rui Moreira.

MS 092 and MS 105 were synthetized from selective inhibitors targeting *Pf*DHODH and GS 082 from inhibitors targeting PDEs, presenting a mechanism of action involving interactions with cell signalling pathway and mitochondrial function.(Harris *et al.*, 2013) All of them chosen according to their activity against the erythrocyte and hepatic stage of the parasite.

Each compound was dissolved in DMSO. For the MS 092, MS105 and GS 082, the dissolution was made to acquire a solution with a final concentration of 5mM. For the chloroquine and atovaquone, the final concentration was 10µM. the solutions were stored at -20ºC until use.

Chloroquine and/or atovaquone were used as the positive controls for all the assays since they are standards drugs used against *P. falciparum* parasites. Chloroquine binds to ferriprotoporphyrin IX, a product of haemoglobin degradation inhibiting hemozoin

polymerization. Atovaquone interferes with parasite mitochondrial electron transport and depolarizes the parasite mitochondria membrane thereby blocking cellular respiration (Farrar et al., 2014).

III.2. BIOLOGICAL MATERIALS

III.2.1. RED BLOOD CELLS SOLUTION

The blood was obtained from healthy voluntary donors with 0^+ erythrocytes from the "Instituto de Higiene e Medicina Tropical". The blood was collected with the help of a vein harvesting system using EDTA (Ethylenediamine tetraacetic acid- an anticoagulant) tubes, by a certified technician from the institute. The collected blood was transferred to falcon tubes and centrifugated at 2500 revolutions per minute (rpm) for 3 minutes (min). The supernatant was discarded, and sterile PBS was added to the pellet to help remove all the plasma and fraction of white blood cells. The centrifugation of the pellet resuspended in sterile PBS, was repeated 5 times to guarantee that only red-blood cells (RBCs are kept. After washing, the red blood cells were stored in a sterile bottle with the same amount of RPMIc to be at 50% haematocrit and were maintained at 4ºC.
This protocol was optimized by our laboratory (Lobo *et al.*, 2018).

III.3. METHODS

III.3.1. *PLASMODIUM FALCIPARUM* IN VITRO CULTURES

- *P. falciparum* line **3D7** (Rosario, 1981) sensitive to chloroquine and mefloquine; a cryo-preserved collection of the UEI Malaria Laboratory/ IHMT;
- *P. falciparum* line **3D7HT-GFP** (MRA-1029, MR4, ATCC® Manassas Virginia); provided by MR4, for distribution by BEI Resources. It is a genetic recombinant constructed by integration of green fluorescent protein (GFP). Deposited by Andrew Talman and Robert Sinden.

The strains were cultivated in cell culture flasks, with 5% haematocrit with 3% parasitaemia. The medium was changed daily to ensure a perfect development of the parasites. The flasks were kept in an incubator with a controlled temperature, (37ºC) and carbon dioxide-CO_2 (5%).

The cultures were monitored daily by smearing on slides fixed with a 100% methanol solution and stained with the Giemsa solution for 20 minutes. The parasites were observed and the parasitaemia was estimated using an optical microscope with a magnification of 100x.

III.3.2. *PLASMODIUM FALCIPARUM* CULTURE SYNCHRONISATION

For some of the tests carried out, the cultures were synchronized to obtain only parasites with a certain age. To achieve this, the cultures were centrifugated at 2500 rpm for 3 minutes. After that, the supernatant was discarder and the pellet resuspended in 6 mL of D-Sorbitol at 5% for 10 min in the incubator. Past 10 min the culture was centrifugated again and the supernatant discarded. The pellet was resuspended in 6mL of sterile PBS to wash out the D-sorbitol and centrifugate once more at 2500 rpm during 3 min. After the wash with sterile PBS the culture was resuspended in RPMIc ensuring that it maintain its 5% haematocrit.

The parasite cultivation, monitorization and synchronization method were optimized by our laboratory (Machado *et al.*, 2016; Lobo *et al.*, 2018).

D-sorbitol acts in the infected RBCs with mature parasites allowing to keep only RBCs infected with parasites in the ring stage. Mature parasites are killed by osmotic shock because the membrane of RBCs infected turns permeable due to structural modifications provoked by mature parasites (Roncalés *et al.*, 2015).

III.3.3. ANTIMALARIAL ACTIVITY OF THE SYNTHETIZED COMPOUNDS

III.3.3.1. SPEED-OF-ACTION ASSAY

The half maximum inhibitory concentration (IC_{50}) value of the compounds was determined using the modified protocols of the IC_{50} speed assay described by Le Manach *et al*., 2013 and the SYBR Green I based fluorescence assay as described previously by Machado *et al.,* 2016. Briefly, 3D7 culture were synchronised with 5% D-Sorbitol to have more than 85% of the parasites in early ring-stage 100µL of a mixture with 3% haematocrit containing 1% of parasitaemia, were distributed in triplicate in a 96-well transparent flat bottom plates. The parasites were submitted to each compound serially diluted (1:3) with concentrations ranging from 10,000 to 0,169 nM and incubated with three different incubations times (24, 48 and 72 hours). After the incubation time, the plates were washed with PBS and SYBR Green dye was added to the parasites for one hour and washed out with PBS at the end. The fluorescence intensity provoked by the SYBR Green dye was read by a multi-mode microplate fluorimeter reader (Triad, Dynex Technologies) with wavelengths excitation of 485 and emission of 535 nm.

To calculate the percentage of survival of the parasites, the fluorescence of each well was calculated relatively to the negative controls (wells with untreated parasites) The IC_{50} value of each compound was esteemed using a nonlinear regression based on a dose-response curve, with the help of the GraphPad Prism software (trial version). To obtain the Speed of Action of the compounds the ratio of IC_{50} at 24hours/ IC_{50} at 72hours was determinate; the result obtained indicated whether the compound was fast or slow acting (close to 1 = fast acting, close to 2 = slow acting).

III.3.3.2. STAGE-SPECIFIC ACTIVITY ASSAY

For the Stage Specific Activity assay the protocol used was optimized by our laboratory based on the Stage Specific analysis as described previously (Le Manach *et al.*, 2013). To perform this essay, the 3D7-GFP culture was synchronised with 5% D-sorbitol twice (6h

hours apart). A mixture with 2% haematocrit and 1% parasitaemia was used for this essay. 100µL of the mix were distributed in triplicate in a 96-well transparent flat bottom plate, were the parasites were challenged with 10x IC_{50} of each compound. The plates were incubated with drugs (CQ, MS92, MS105, GS82) for 24 hours. After the 24h the medium was changed with a new medium without drugs. After 24 hours without drug, the mixture was diluted by 1/16 with 2% haematocrit and incubated again for 96 hours with a medium change after the first 48 hours. The survival and the viability of the parasites were evaluated by microscopy and cytometry at the beginning (T0), after the first 48 hours (T48) and at the end (T96) of the experiment.

For the microscopic evaluation, a smear of the culture, at t 0h, t 48h and t 96h, was done and coloured with Giemsa and read on an optical microscope with a magnification of 100x.

The cytometer measurements performed on a CytoFLEX Blue-Red-Violet (B-R-V) Flow Cytometer (Beckman Coulter) equipped with 405 nm, 488 nm and 633 nm lasers. The following channels and probes were used: green (CM-H_2DCFDA; 492 - 495 nm excitation, emission 517 - 527 nm bandpass) and red (SYTO^{TM} 61; 628 nm excitation, emission 645 nm bandpass). Samples were diluted to 0.5 - 1% hematocrit and 10 000 events (encompassing uninfected and infected RBCs) were acquired based on the forward *versus* side scatter profiles. Detector gain settings varied between experiments to optimize signals but were kept constant within individual experiments and no compensation was applied to any of the channels. Analysis was performed using FlowJo Vx0.7.

This experiment could have been done using the 3D7 culture, labelling it with SYBR Green dye. We choose to use the 3D7-GFP line because its an easy and efficient way to track the parasite throughout its life stages without interfering in its survival (Talman, Blagborough and Sinden, 2010).

III.3.3.3. REACTIVE OXYGEN SPECIES QUANTIFICATION ASSAY

To determine the reactive oxygen species (ROS), a mixture with 5% parasitaemia and 2% haematocrit was prepared using a 3D7 culture previously synchronized with 5% D-sorbitol to begin the experience with more than 80% of the parasites in the trophozoite

and schizont stages. The protocol followed was optimized by our laboratory based on the literatures(Fu *et al.*, 2010; Mohring, Jortzik and Becker, 2016). More precisely, 200 µL of the mixture was distributed in eppendorf tubes where drug treatment was carried out for 4 h with $10\times IC_{50}$ of each compound to be tested. Chloroquine and hydrogen peroxide (H_2O_2 at 10 mM) were used as positive controls. After 4h incubation 2,5µM CM-H_2DCFDA, and 0,5µM SYTO 61, was added to the mixture and incubated for more 30 min at 37ºC and 5% CO_2. The eppendorf tubes were removed from the incubator and centrifugated during 1 min at 2800 rpm and washed with pbs twice. The oxidative stress was measured by a cytometer and the results analysed using the FlowJo software.

III.3.3.4. MITOCHONDRIAL MEMBRANE POTENTIAL ASSAY

The evaluation of the mitochondrial membrane potential was realised challenging an asynchronous mixture of 3D7 culture, with 5% parasitaemia and 2% haematocrit, for six hours. The mixture was distributed in eppendorf tubes and 10µM of each compound to be tested was added. Atovaquone was used as positive controls. After the incubation time, 5 µM Rhodamine 123 were added to the mixture and incubated for 5 min. Past 5 min, the tubes were centrifugated at 2800 rpm for 1 min and washed with PBS. The pellet was resuspended in PBS and distributed in a 96-well black flat bottom plates and the fluorescence read by the multi-mode microplate fluorimeter reader (Triad, Dynex Technologies) (Biagini *et al.*, 2006; Antoine *et al.*, 2014).

IV-RESULTS AND DISCUSSION

IV.1. ANTIMALARIAL ACTIVITY OF THE SYNTHETIZED COMPOUNDS

IV.1.1. SPEED-OF-ACTION ASSAY

To better characterize the antimalarial activity of the compounds, first, a speed-of-action essay was performed. A half maximum inhibitory concentration (IC_{50}) value was determined, at different incubation times (figure IV.1) and used to determinate the ratio of IC_{50} 24h/48h and IC_{50} 24h/72h of the controls and compounds. This essay was done three time in triplicates and results obtained are represented in figure IV.2-A. The IC_{50} values obtained for the control drug, CQ, was

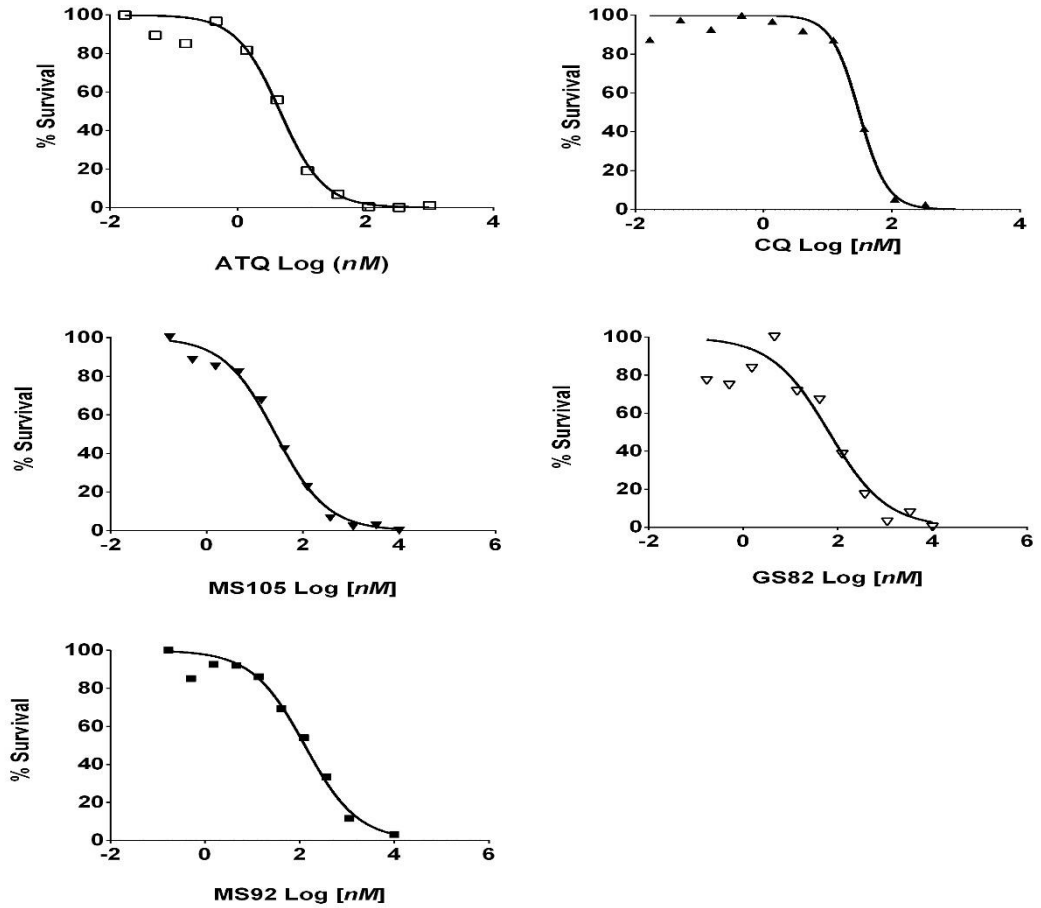

Figure IV.1 Dose-response curves to estimate the IC_{50} of tested compounds and control drugs against *Plasmodium falciparum*. ATQ, Atovaquone; CQ, chloroquine; MS105, GS92, MS92 newly synthetized compounds.

similar at the different incubation times, with ratio of 1,0 for both IC_{50}24h/IC_{50}48h and IC_{50}24h/ IC_{50}72h. These ratios are in accordance with literature (Le Manach *et al.*, 2013). These results were expected, since CQ is known to be a fast-acting drug, that is, it has a fast parasite clearance time (C. D. Fitch; D. C. Warhurst, 1986). For the other control drug, ATQ, the IC50 values obtained were similar after 24 hours and 48 hours incubations and different after 72h (p=0,0010; Mann-Whitney) (figure IV.2-B). Hence, for ATQ, the correspondent ratios were 1,0 for IC_{50}24h/ IC_{50}48h and 2.6 for IC_{50}24h/ IC_{50}72h (figure IV.2-A).

A)

	IC50 (nM)			ratio IC50	
	24h	48h	72h	24h/ 48h	24h/ 72h
	Mean ± SD	Mean ± SD	Mean ± SD		
CQ	21,2 ± 3,0	22,0 ± 1,9	20,4 ± 3,7	1,0	1,0
ATQ	2,9 ± 0,3	3,0 ± 1,3	1,1 ± 0,6	1,0	2,6
GS82	66,1 ± 18,6	66,7 ± 6,5	96,6 ± 18,2	1,0	0,7
MS92	23,3 ± 5,7	51,3 ± 26,6	49,6 ± 23,0	0,5	0,5
MS105	26,0 ± 0,3	29,6 ± 16,4	26,4 ± 7,6	0,9	1,0

B)

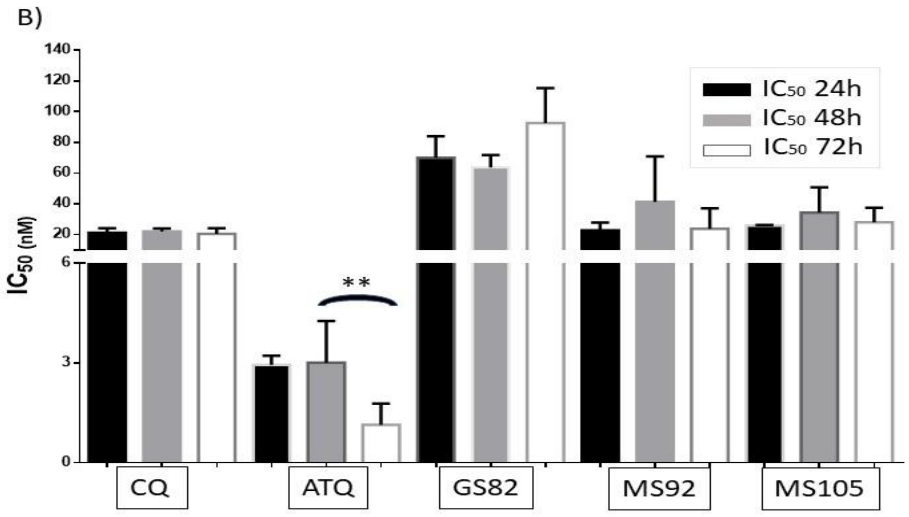

Figure IV.2. Speed-of-action of the diferent compounds using *Plasmodium falciparum* 3D7 strain. A) IC_{50} values determined at diferent incubation times (24h, 48h and 72h) and the IC_{50} ratios. B) graphical representation of the IC_{50} values of the compounds (MS92, MS105, GS82) and the control drugs; chloroquine (CQ)and atovaquone (ATQ), at 24h, 48h and 72h incubations. ** p=0,0010; Mann Whitney test.

ATQ is known to be a slow-acting drug as it need more time in contact with the parasite to eliminate it from the bloodstream (Nixon *et al.*, 2013), this behaviour was reflected by the ratio 24h/72h obtained (figure IV.2-A) and is in agreement with other authors (Sanz *et al.*, 2012; Le Manach *et al.*, 2013; Wilson *et al.*, 2013). However, the 24h/48h ratio was ± 1 (figure IV.2-A), a value expected from a fast, rather than a slow-acting drugs (Wein *et al.*, 2010; Sanz *et al.*, 2012; Le Manach *et al.*, 2013). This might mean that isolated, the ratio 24h/48h is not potent enough to discriminate slow from fast acting drugs.

Regarding the tested compounds, MS105 presented an IC_{50}24h/IC_{50}72h ratio of 1,0 whereas MS92 and GS82 presented an IC_{50}24h/IC_{50}72h ratio below 1(figure IV.2-A).
The IC_{50}24h/IC_{50}72h ratio value obtained for the MS105 compound indicate that it can be considered as a fast-acting compound (Sanz *et al.*, 2012; Le Manach *et al.*, 2013) since it is similar to the IC_{50}24h/IC_{50}72h ratio obtained for CQ.
The IC_{50}24h/IC_{50}72h ratio values obtained for the MS92 and GS82 compounds remained below 1, indicating that this result alone may be inconclusive to determinate the speed-of-action of these two compounds.
The elevated standard deviation (figure IV.2-A), obtained in the IC_{50} values of the compounds (GS82, MS92,MS105) can be explained by the fact that these compounds are new synthetized compounds and they might not be as stable as the controls.

IV.2. STAGE-SPECIFIC ASSAY

The second experiment to characterize the antimalarial activity of the synthetised compounds was to determinate the stage-specif activity. The aim of this essay was to determinate if the compounds where more active against a particular stage of development of the parasite. As described earlier, the 3D7-GFP culture used for this experiment was synchronized with sorbitol, in order to obtain cultures enriched with rings or trophozoits at the beginning of the experiment.

A) OPTICAL MICROSCOPY *VS* FLOW CYTOMETRY

Synchronization was verified and parasitemia determined in paralel by optical microscopy and flow cytometry analysis after incubation times (t0h and t48h).

Histogram of (green GFP fluorescence) synchronized cultures of *Plasmodium falciparum* strain 3D7-GFP, after 48 hours incubation (t48h), under standard culture conditions and the respcetive picture of the diferent stages are presented in figure IV.3. The histogram,

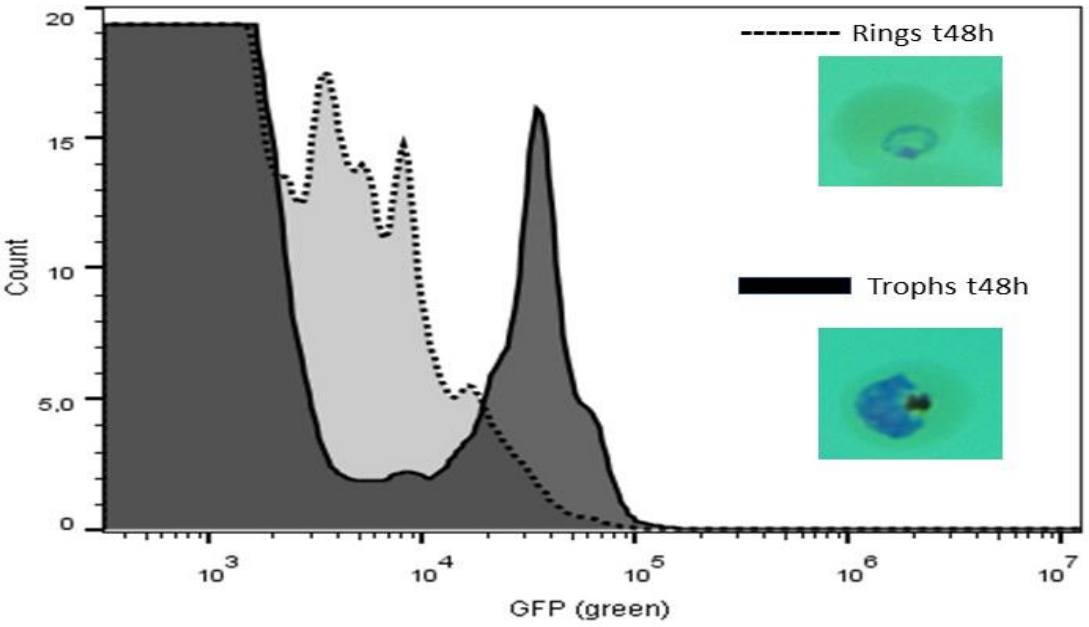

Figure IV.3. **Flow cytometry histograms for ring- and trophozoite-stage synchronized cultures of *P. falciparum* strain 3D7-GFP strain.** Dotted black line, ring-stage parasites; Solid black line, trophozoites-stage parasites. Insert pictures of ring- and trophozoite-stage parasites after 48 hours incubation (t48h), under standard culture conditions.

made using the results obtained using the cytometer and analised with the Flowjo Vx 0,7 software, show the fluorescence intensity emitted by the diferent stages of the parasite. It can be observed that the parasites in the ring-stage, had lower fluorescence, close to the unparasited red blood cells (RBCs). The parasites in the trophozoite-stage, showed a higher green fluorescence. The fluorescence intensity of the trophozoite-stage parasites were about 10 fold higher than those of the ring-stage parasites as described in the literature (Schuck *et al.*, 2011).

Parasitaemia obtained by optical microscopy and flow cytometry analysis after the same incubation time (t0h and t48h) were compared in order to validate the cytometer analises and poursuite the examination of the results focussing only on the cytometer results. These parasitaemias obtained by optical microscopy and flow cytometry are presented in table IV.1

Table IV.1. Parasitaemia obtained by optical microscopy and flow cytometry.

% Parasitaemia	Rings		Trophozoites	
	Cyt.	OM.	Cyt.	OM.
t 0h	1,1	1,0	1,1	1,0
t 48h	4,6	4,5	4,7	4,5

Cyt. – flow cytometry; OM. – optical microscopy.

From the values presented in table IV.1, we concluded that, the % of parasitaemia determined by optical microscopy or by flow cytometry were identical, hence alowing us to use flow cytometry to study the stage specific effect of the new compounds.

B) STAGE-SPECIFIC ACTIVITY DETERMINATION

Histogram of (green GFP fluorescence) synchronized cultures of *Plasmodium falciparum* strain 3D7-GFP, after 48 hours incubation (t48h), under standard culture conditions, presented in figure IV.4, shows the fluorescence intensity emitted by the different stages of the parasite after challenge with the compounds (MS92; MS105; GS82) and the control drug chloroquine. It can be observed that, parasites challenged with CQ had the lowest fluorescence intensity in both stages. The ring-stages parasites challenged with the

compounds had higher fluorescence intensity but lower than the untreated parasites (figure IV.4-A). The trophozoite-stage parasites challenged with the compounds MS92 and MS105 show similar fluorescence intensity between them, but lower than the fluorescence intensity of the untreated parasites. The parasites challenged with the compound GS82 show a fluorescence intensity similar to those of the untreated parasites (figure IV.4-B).

The efficacy of the compounds was analysed using the Mann-Whitney test and presented in figure IV.5.

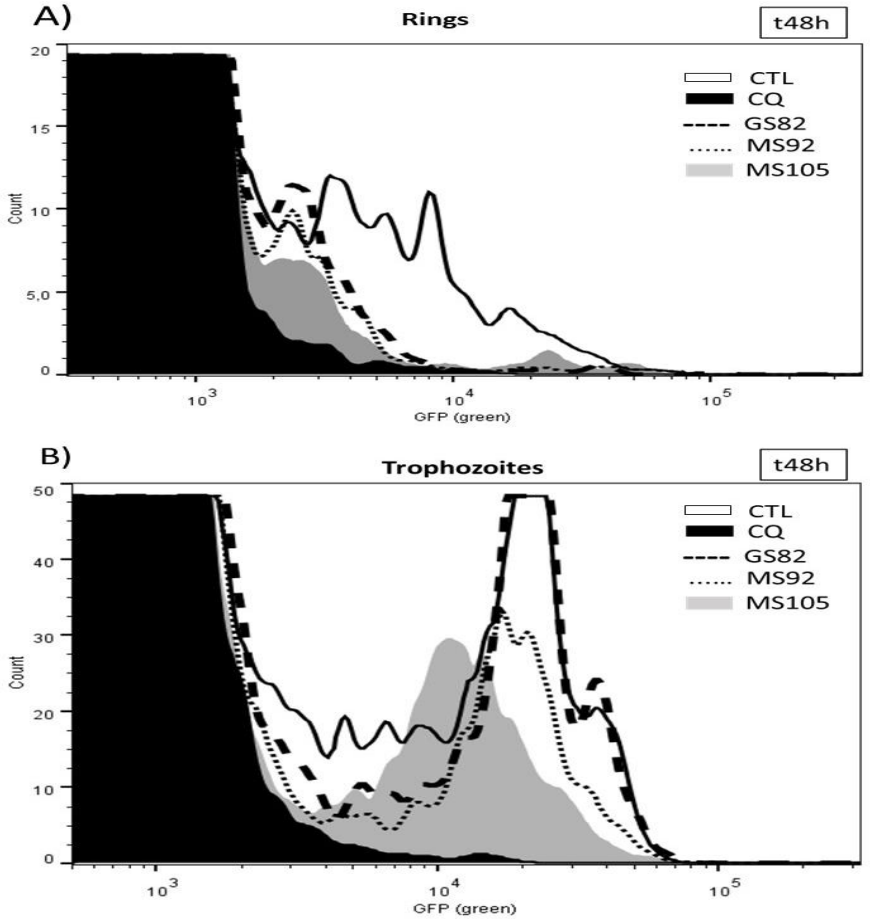

Figure IV.4. Flow cytometry histograms of stage dependent effects of the compounds and chloroquine. Histograms of (green GFP fluorescence) synchronized cultures of *Plasmodium falciparum* after 48h (t 48h) incubation time. A) ring-stage parasites. B) trophozoite-stages parasites. Filled black, CQ; filled grey, MS105; dotted black line, MS92; heavy dashed black line, GS82, solid black line; CTL.

Chloroquine showed to have similar activity against ring- and trophozoite-stage parasites after 48 hours incubation (t 48h), presenting an efficacy of 88% in both stages of the parasite (figure IV.5). This behaviour was expected since CQ is known to influence the growth of all parasites stages similarly (Maerki *et al.*, 2006; Le Manach *et al.*, 2013).
The compound MS92 showed a stage-specific profile of activity similar to CQ, with a similar activity against both stages of the parasite (figure IV.5).
The compounds MS105 and GS82, however, presented higher activity against ring-stage parasites (p= 0,0173; p= 0,0022; Mann-Whitney respectively) (figure IV.5).

There was no significant difference between the two potential *Pf*PDE inhibitors activity against the parasites. MS92 and MS105 were more active against both parasite stages (p= 0,0043; Mann-Whitney) than the potential *Pf*DHODH inhibitor compound GS82 (figure IV.5).

From the literature, it's known that the *P. falciparum* genome encodes two PDEs (*Pf*PDEα and *Pf*PDEβ) that are expressed in asexual blood stage reproduction (Baker *et al.*, 2017b), but there is no knowledge in which stage of the parasite they're expressed yet. Given the fact that these two compounds (MS92 and MS105), that were synthesized to be phosphodiesterase (PDEs) inhibitors, acts against both stages of the parasite, corroborates with the literature. The fact that the compound MS105 is more active against the ring-stage parasite, give new insights into the mode of action of the compound.

It is known from the literature that the *Pf*DHODH is essential for the cell growth of the parasite (Phillips and Rathod, 2010), but there is no knowledge if they have a particular action in the stages development of the parasite. The fact that the compound GS82 (a potential *Pf*DHODH inhibitor), showed a predominant activity against the ring-stage parasites (p= 0,0022; Mann-Whitney) (figure IV.5), suggest that the mode of action of this compound is suitable for further experiments to confirm its activity. Being active against the ring-stage parasites could be a suitable characteristic since there is evidence that resistances against antimalarial drugs may occurs in the ring-stages parasites (Blasco, Leroy and Fidock, 2017).

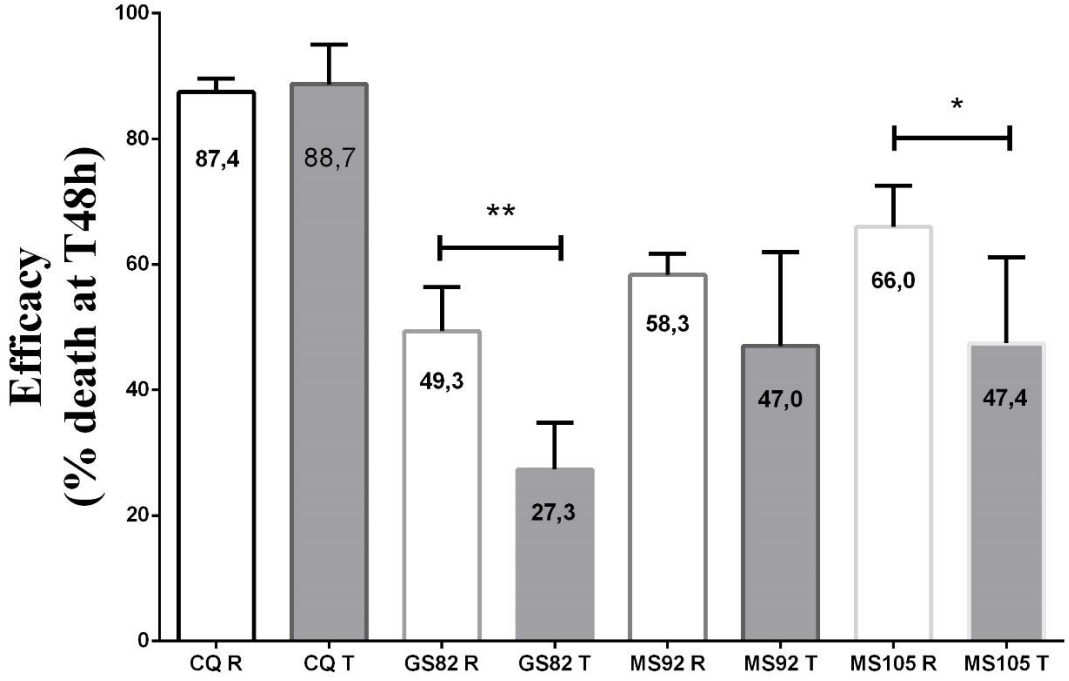

Figure IV.5. Stage dependent effect of the new compounds and chloroquine, on synchronous culture of *Plasmodium falciparum* strain 3D7-GFP at t48h. Compound efficacy are expressed as the percentage of death relative to an untreated control. CQ, chloroquine; GS82-MS92-MS105, new compounds. R, ring-stage parasites (empty bars); T, trophozoite-stage parasites (filled bars). ** P=0,0022; * P=0,0173. Mann-Whitney test.

C) CYTOCIDAL *VS* CYTOSTATIC EFFECT OF COMPOUNDS

Cultures of *Plasmodium falciparum* strain 3D7-GFP were challenged with CQ and the compounds (MS92, MS105, GS82) for 24 hours. After 24h, the medium was changed with new medium without drug and incubated for more 24h. After which the cultures were incubated for additional 96 hours as indicated in section III.3.3.2. Extending the stage-specific assay until 96 hours (t96h) allowed us to estimate if compounds had cytocidal or cytostatic effect on the parasite growth, by analysing whether the parasites

were able to recover from the damage caused by the compounds (Michelle F Paguio, Bogle and Roepe, 2011).

Histogram of (green GFP fluorescence) synchronized cultures of *Plasmodium falciparum* strain 3D7-GFP, after 96 hours incubation (t96h), under standard culture conditions, presented in figure IV.5. The histogram shows the fluorescence intensity emitted by the different stages of the parasite after changing the medium, allowing them to grow with no constraint. It can be observed that, the parasites previously challenged with CQ, had the lower fluorescence intensity in both stages of the parasite. The ring-stages parasites that were previously challenged with the compounds (MS92; MS105; GS82) had higher fluorescence intensity than the parasites challenged with CQ, but lower than the untreated parasites (figure IV.6-A). The trophozoite-stages parasites challenged with the compounds MS92 and MS105 show similar fluorescence intensity between them, but lower than the untreated parasites. The parasites challenged with the compound GS82, on the other hand, show a fluorescence intensity similar to the untreated parasites (figure IV.6-B).

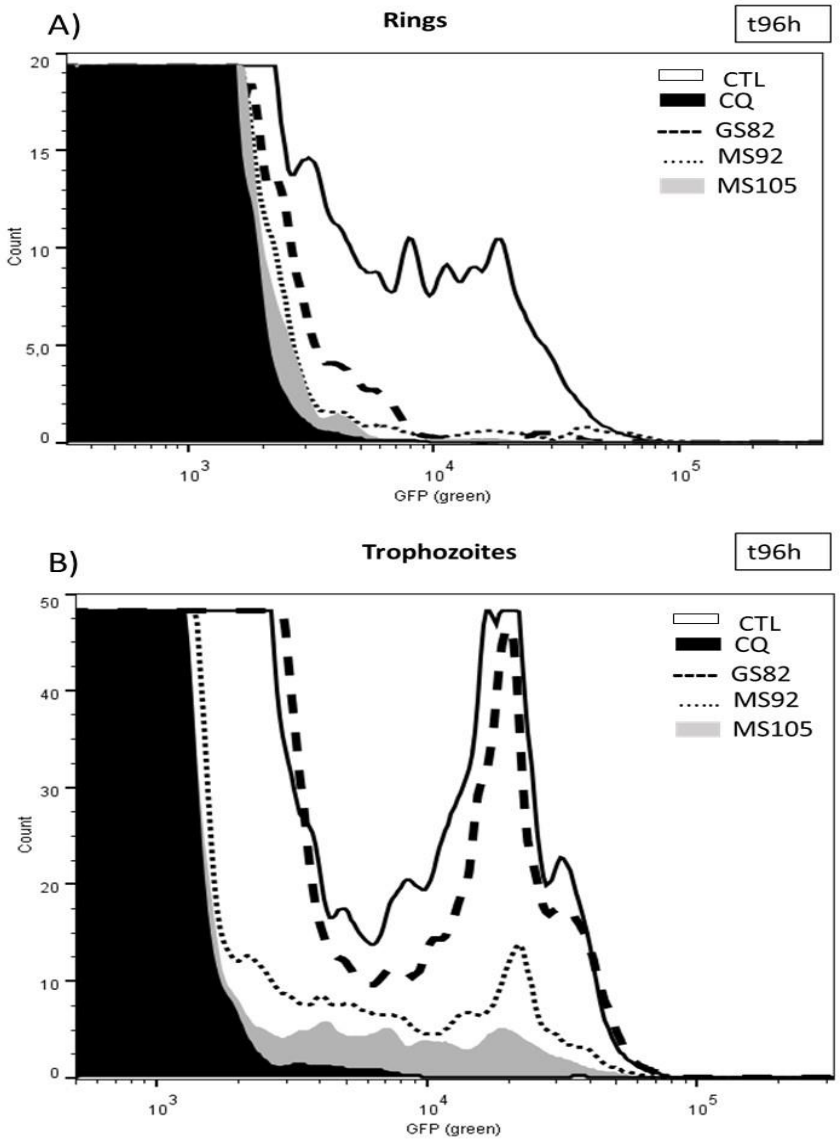

Figure IV.6. Flow cytometry histograms of stage dependent effect of the compounds and chloroquine. Histograms of (green GFP fluorescence) synchronized cultures of *Plasmodium falciparum* after 96h. t 96h, incubation time; filled black, CQ; filled grey, MS105; dotted black line, MS92; heavy dashed black line, GS82; solid black line; CTL (untreated culture).

The efficacy of the compounds was analysed using the Unpaired t test and presented in figure IV.7.

After 96 hours incubation without drug, we could observe that chloroquine had an efficacy > 95% in the ring- and trophozoite-stage of the parasites. This behaviour was expected since CQ is known to be a cytocidal drug and that can to induce growth

inhibition and irreversible morphological changes in both stages of the parasite (Maerki *et al.*, 2006; Le Manach *et al.*, 2013; Roepe, 2014).

The compound MS105 presented an efficacy against the ring-stage parasites (± 94%), similar to CQ (figure IV.7). Its efficacy against the trophozoite-stage parasite was a little bit inferior (83 %) (p=0,0238, Mann-Whitney). Considering the low percentage(< 20%) of parasites of both stages that manage to recover from drug treatment (Michelle F Paguio, Bogle and Roepe, 2011) at t96h, we can conclude that MS105 had a more cytocidal effect.

Regarding the effect of the compound MS92, we could observe that it was a little more efficient against the ring-stage parasite (± 89%) than against the trophozoite-stage parasites (±77%), (p=0,0238, Mann-Whitney) (figure IV.7). It can be considered a cytocidal compound since most parasites (> 75%) (Michelle F. Paguio, Bogle and Roepe, 2011) couldn't recover after the drug treatment.

Compounds MS92 and MS105 presented no difference between their toxicity against the trophozoite-stage of the parasite, however, MS105 was more toxic to ring-stage parasites (p=0,00021, Unpaired t test) than MS92 (figure IV.7).

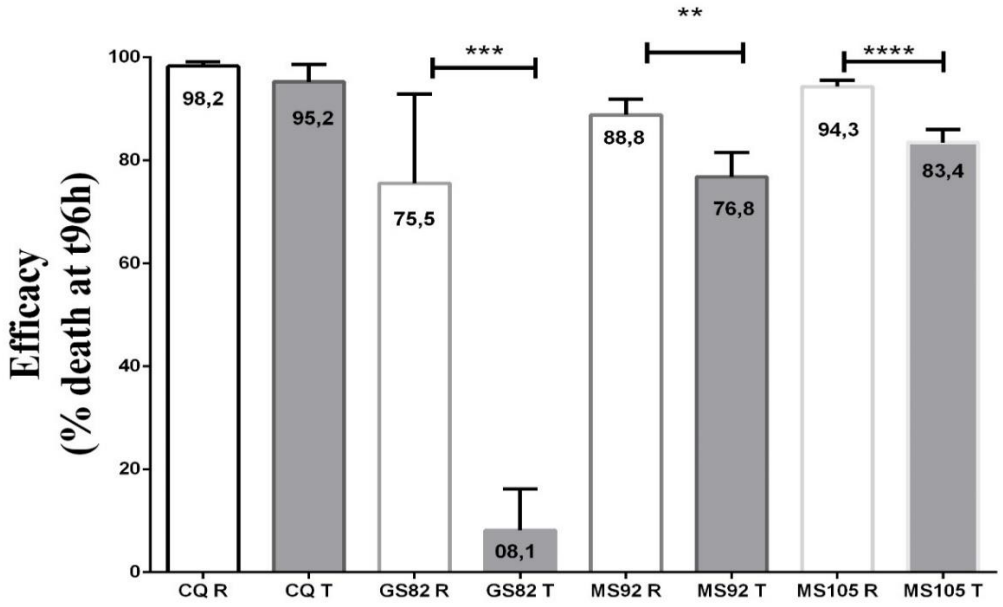

Figure IV.7. Stage dependent effect of the new compounds and chloroquine on synchronous culture of *Plasmodium falciparum* strain 3D7-GFP after 96 hours. Compound efficacy are expressed as the percentage of death relative to an untreated control. The empty bars represent the ring-stage parasites (R) and the filled bars represent the trophozoite-stage parasites (T). *** p=0,0004; ** p=0,00021; **** p<0,0001. Unpaired t test.

Compound GS82, presented higher toxic activity against ring- (> 75%) parasites (p=0.0004, Unpaired t test) than against trophozoite-stage (< 10%) parasites (figure IV7) at t96h. This compound seems to have a variable toxicity depending on the stage of the parasite: cytocidal against the ring-stage parasites and cytostatic against the trophozoite-stage parasites. When a compound has different effect on different stages of the parasite, it can be due to the dose of the compound used, or due to the age of the parasite used at the begin of the experiment (Roepe, 2014).

Analysing all the compounds regarding their toxicity, it appears that the potential *Pf*PDE inhibitors (MS92 and MS105) exerted a cytocidal effect against both stages of the parasite, on the other hand the *Pf*DHODH inhibitor appear to be cytocidal against the ring-stage parasites and presenting a more cytostatic effect against the trophozoite-stages parasites. Most of the trophozoite-stage parasites were able to recover after the contact with GS82 (figure 7).

IV.3. REACTIVE OXYGEN SPECIES QUANTIFICATION ASSAY

For the determination of the reactive oxygen species (ROS) assay, CM-H_2DCFDA was combined with SYTO 61 (nucleic acid dye) to monitor oxidative stress variation at different parasite-stages as it is performed and validated in the literature (Mohring, Jortzik and Becker, 2016; Mohring *et al.*, 2017; Gunjan *et al.*, 2018). When the reduced form of CM-H_2DCFDA is added to cells, it diffuses freely across membranes. Oxidation of H_2DCF by reactive oxygen species (especially hydrogen peroxide in the presence of iron or hematin) produces highly fluorescent 2',7'dichlorofluorescein (here on designated as DCF)(Fu *et al.*, 2010).

Asynchronous cultures (*P. falciparum* 3D7) where treated as described in section III.3.3.3. Results were analysed by flow cytometry and reinforced by microscopic analysis. No compensation was need when using the flow cytometry since the dyes used for this experiment were different and there was no overlap in the detector of the colours used (Roederer, 2001).

Analysing the results obtained using untreated parasites, we could identify the DCF fluorescence of the parasites based on the 2-dimensional plots of DCF (green) and SYTO 61 (red) fluorescence. Gates were set in mode to differentiate the uninfected RBCs population, the ring-stage and the trophozoite-stage population.

Initially we labelled the parasites with only DCF, then only with SYTO 61 and finally with DCF and SYTO 61 as demonstrated in figure IV.8. The parasites labelled with only DCF (figure IV.8-a), exhibit a low green signal but show a small population (Q1) exhibiting increased DCF fluorescence. When labelled with SYTO 61 alone. a population with different levels of red signal are distinguished but all exhibit a low green signal (Q3) (Figure IV.8 b). When labelled with DCF and SYTO 61, a small unsynchronised population exhibiting different levels of red and green signal can be observed (Q2 and Q3) (figure IV.8-c). It is related in the literature, (Fu *et al.*, 2010; Mohring, Jortzik and Becker, 2016), that the ring-stage (Q3) parasites exhibit a DCF signal that is similar to that of the uninfected RBCs (Q4), whereas the trophozoite-stage (Q2) parasites are

associated with a greater DCF signal and in uninfected cultures both the red and green signals are low (Q4) (figure IV.8 a, b and c). Even though the parasites observed are unsynchronized, the gates help us to differentiate the different stages of the parasites and confirm that the results obtained are in line with what was observed in the literature.

Considering that the trophozoite-stage parasites are associated a greater DCF signal, it would be suitable for monitorization of oxidative stress variation caused by drugs to be done using preferentially trophozoite-stages parasite so the results can be more consistent and precise.

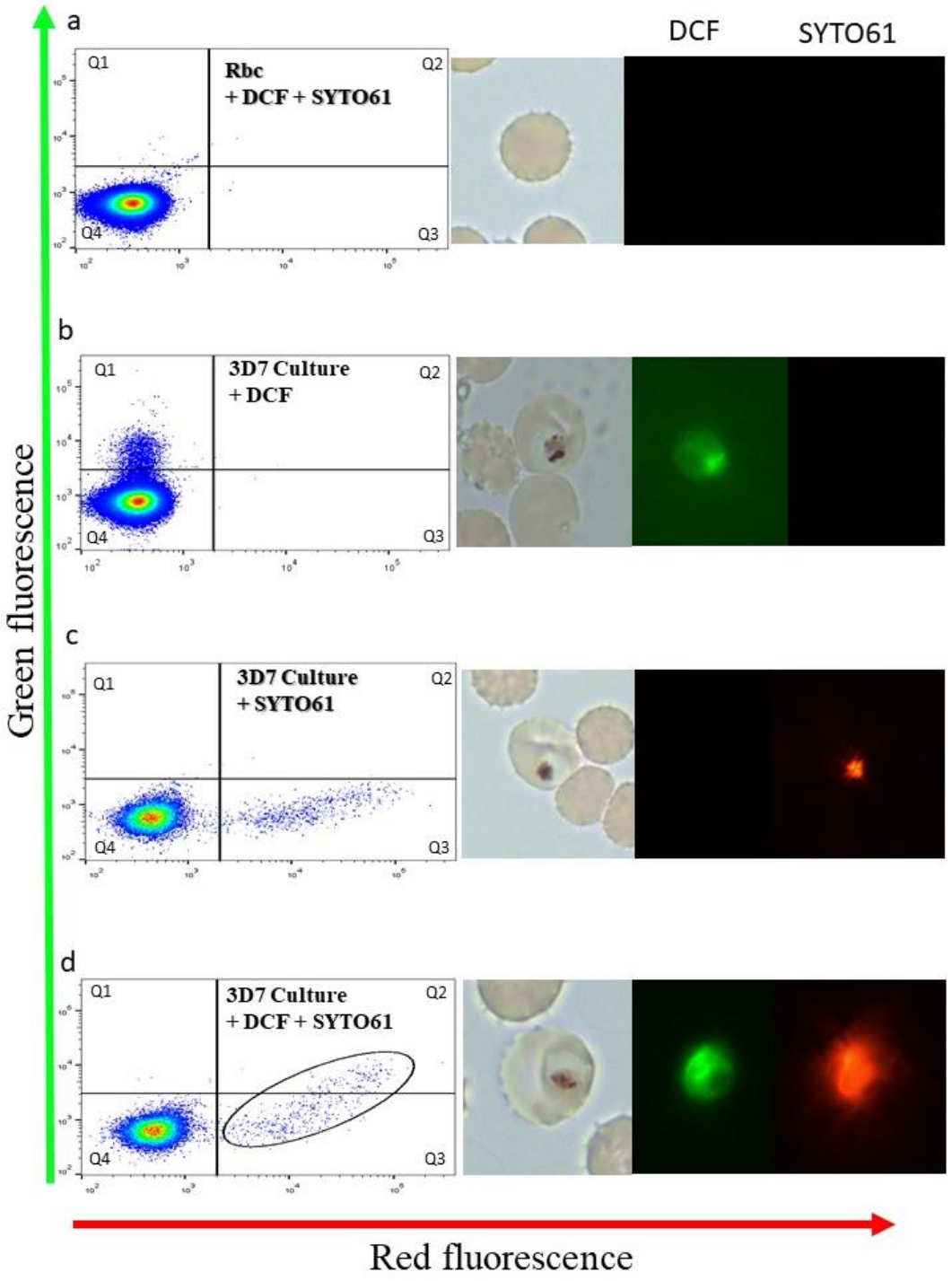

Figure IV.8. DCF signals in the ring and trophozoite stages parasites. Red blood cells labelled with CM-H_2DCFDA and SYTO 61(a) and asynchronous 3D7 cultures labelled with CM-H_2DCFDA (b), SYTO 61 (c), CM-H_2DCFDA and SYTO 61 (d), (as indicated in each panel), analysed by flow cytometry and imaged using a fluorescence microscope. Two-dimension plots of the red and green fluorescence intensity are presented.

IV.4. MITOCHONDRIAL MEMBRANE POTENTIAL ASSAY

For the evaluation of the mitochondrial membrane potential assay, atovaquone (ATQ) was used as control drug because of it known activity against the mitochondria by inhibiting the mitochondrial electron transport and depolarization of mitochondrial membrane, causing death of the parasite (Srivastava, Rottenberg and Vaidya, 1997; Biagini *et al.*, 2006; Nixon *et al.*, 2013). Rhodamine 123 (a cationic fluorescent dye) was used to monitor the membrane potential of the mitochondria. The dye distributes according to the negative membrane potential across the mitochondrial inner membrane. The loss of membrane potential results in the loss of the dye and, therefore, the fluorescence intensity (Chazotte, 2011; Antoine *et al.*, 2014).
Atovaquone, the selective bc1 complex inhibitor, showed a decrease of the total membrane potential dependent fluorescence by proximally 16% (figure IV.8). This behaviour was expect since its known in the literature that ATQ can reduce the total membrane potential dependent fluorescence up to 30% (Biagini *et al.*, 2006; Antoine *et al.*, 2014).

The compound MS92 showed a decrease of fluorescence of ± 13%, while the compound MS105 and GS82 showed a decrease of ± 7% and ± 9% respectively (figure IV.9). The compound MS92 showed to have an activity more similar to ATQ despite the significative statistical difference observed (p=0,0411, Mann-Whitney).

Results obtained by the compound MS92 (a potential *Pf*PDE inhibitor) could be expected, since several studies suggested that PDEs have an intramitochondrial activity (Acin-Perez *et al.*, 2009, 2011; Monterisi *et al.*, 2017). Since this compound is the only one to show results more similar to ATQ, suggest that MS92 could have an important role in the parasite mitochondria. The fact that MS105 (a potential *Pf*PDE inhibitor too) showed small decrease effect in the membrane potential, suggest that only a specific *Pf*PDE inhibitor can impact the mitochondria *Pf*PDE's activity.

According to the literature, DHODH can be found in the mitochondrial intermembrane space (Fang *et al.*, 2013), it was expected that the compound GS82 (a potential *Pf*DHODH

inhibitor) provoked a higher decrease of the membrane potential dependent fluorescence. That was not observed, GS82 induced a small membrane potential dependent fluorescence decrease (Fig. IV.9).

These methodologies showed to be adequate to estimate the mitochondrial membrane potential interference of new compounds, but more experiments are needed to confirm the compounds behaviour.

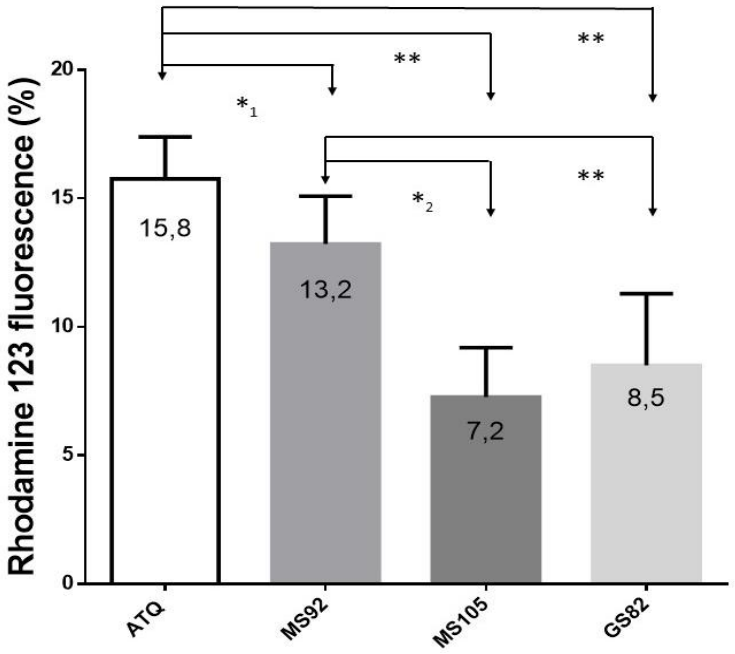

Figure IV.9. Effect of Atovaquone (ATQ) and the synthetized compounds (GS82- MS92- MS105) on mitochondrial membrane potential of *Plasmodium falciparum* strain 3D7. Each bar represents the mean from experiments performed independently ± standard errors, done in triplicate (n=6). **P=0,0022; *1 P=0,0411; *2 P=0,0152. Mann-Whitney test.

V-CONCLUSIONS

V-CONCLUSSIONS

Regarding the speed-of-action assay, we could conclude that of the three compounds tested, the compound MS105 was the only one to present a fast-acting profile. The results obtained for the compounds MS92 and GS82 suggest that they are slow-acting compounds.

When analysing the stage-specific essay, we could conclude that of the three compounds, the compound MS105 had a higher activity against the ring-stages parasites, and GS82 had a similar activity against both stages of the parasite. MS92 and MS105 have a cytocidal activity profile while GS82 seem to have a cytocidal effect against the ring-stage parasite, and a cytostatic effect against the trophozoite-stage parasites.

Combining the speed-of-action and the stage-specific assays, described here, we have a valid and fast approach to discriminate between fast- and- slow-acting, cytocidal and cytostatic antimalarials compounds, providing valuable information to guide and accelerate the development of new classes of antimalarial compounds.

The optimized ROS protocol demonstrate that, using CM-H_2DCFDA to assess oxidative stress in the parasites, combined with SYTO 61, is reliable. The results obtained here are in line with previous observations and suggests that the combination of these two fluorescence-based probes, could facilitate the monitorization of the effects of different metabolic inhibitors and provide more insight mechanisms of anti-malarial drug action.

The optimized mitochondrial membrane potential assay showed that the results for atovaquone were in line with previous observations. Suggesting that the use of Rhodamine 123 to monitor the membrane potential is consistent and can provide more insights into the mechanism of anti-malarial drug action.

VI-REFERENCES

VI-REFERENCES

Acin-Perez, R. *et al.* (2009) 'Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation.', *Cell metabolism*. NIH Public Access, 9(3), pp. 265–76. doi: 10.1016/j.cmet.2009.01.012.

Acin-Perez, R. *et al.* (2011) 'Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation.', *Cell metabolism*. Elsevier, 13(6), pp. 712–9. doi: 10.1016/j.cmet.2011.03.024.

Amaratunga, C. *et al.* (2016) 'Dihydroartemisinin–piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study', *The Lancet Infectious Diseases*, 16(3), pp. 357–365. doi: 10.1016/S1473-3099(15)00487-9.

Antinori, S. *et al.* (2012) 'Biology of human malaria plasmodia including Plasmodium knowlesi.', *Mediterranean journal of hematology and infectious diseases*. Catholic University in Rome, 4(1), p. e2012013. doi: 10.4084/MJHID.2012.013.

Antoine, T. *et al.* (2014) 'Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential.', *The Journal of antimicrobial chemotherapy*. Oxford University Press, 69(4), pp. 1005–16. doi: 10.1093/jac/dkt486.

Ashley, E. *et al.* (2006) 'Malaria', *Travel Medicine and Infectious Disease*. Elsevier, 4(3–4), pp. 159–173. doi: 10.1016/J.TMAID.2005.06.009.

Ashley, E. A. and Phyo, A. P. (2018) 'Drugs in Development for Malaria', *Drugs*. Springer International Publishing, pp. 1–19. doi: 10.1007/s40265-018-0911-9.

Baird, J. K. (2011) 'Resistance to chloroquine unhinges vivax malaria therapeutics.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 55(5), pp. 1827–30. doi: 10.1128/AAC.01296-10.

Baker, D. A. *et al.* (2017a) 'Cyclic nucleotide signalling in malaria parasites.', *Open biology*. The Royal Society, 7(12). doi: 10.1098/rsob.170213.

Baker, D. A. *et al.* (2017b) 'Cyclic nucleotide signalling in malaria parasites.', *Open biology*. The Royal Society, 7(12). doi: 10.1098/rsob.170213.

VI-REFERENCES

Barnes, K. I. *et al.* (2008) 'Increased Gametocytemia after Treatment: An Early Parasitological Indicator of Emerging Sulfadoxine-Pyrimethamine Resistance in Falciparum Malaria', *The Journal of Infectious Diseases*. Oxford University Press, 197(11), pp. 1605–1613. doi: 10.1086/587645.

Beraldo, F. H. *et al.* (2005) 'Cyclic AMP and calcium interplay as second messengers in melatonin-dependent regulation of Plasmodium falciparum cell cycle.', *The Journal of cell biology*. The Rockefeller University Press, 170(4), pp. 551–7. doi: 10.1083/jcb.200505117.

Biagini, G. A. *et al.* (2006) 'Functional characterization and target validation of alternative complex I of Plasmodium falciparum mitochondria.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology, 50(5), pp. 1841–51. doi: 10.1128/AAC.50.5.1841-1851.2006.

Blasco, B., Leroy, D. and Fidock, D. A. (2017) 'Antimalarial drug resistance: linking Plasmodium falciparum parasite biology to the clinic.', *Nature medicine*. NIH Public Access, 23(8), pp. 917–928. doi: 10.1038/nm.4381.

Burrows, J. N. *et al.* (2013) 'Designing the next generation of medicines for malaria control and eradication', *Malaria Journal*, 12(1), p. 187. doi: 10.1186/1475-2875-12-187.

Butler, A. R., Khan, S. and Ferguson, E. (2010) 'A brief history of malaria chemotherapy', *Journal of the Royal College of Physicians of Edinburgh*. doi: 10.4997/JRCPE.2010.216.

C. D. Fitch; D. C. Warhurst (1986) 'The mode of action of chloroquine and related malarial schizontocides', *Parasitology Today*, 2(12), p. 329. doi: 10.1016/0169-4758(86)90050-5.

Carrara, V. I. *et al.* (2013) 'Malaria Burden and Artemisinin Resistance in the Mobile and Migrant Population on the Thai–Myanmar Border, 1999–2011: An Observational Study', *PLoS Medicine*. Edited by N. Ford, 10(3), p. e1001398. doi: 10.1371/journal.pmed.1001398.

Chazotte, B. (2011) 'Labeling mitochondria with rhodamine 123.', *Cold Spring Harbor protocols*. Cold Spring Harbor Laboratory Press, 2011(7), pp. 892–4. doi:

10.1101/pdb.prot5640.

Cowman, A. F. *et al.* (2016) 'Malaria: Biology and Disease.', *Cell*. Elsevier, 167(3), pp. 610–624. doi: 10.1016/j.cell.2016.07.055.

Cui, L. *et al.* (2015) 'Antimalarial Drug Resistance: Literature Review and Activities and Findings of the ICEMR Network.', *The American journal of tropical medicine and hygiene*. The American Society of Tropical Medicine and Hygiene, 93(3 Suppl), pp. 57–68. doi: 10.4269/ajtmh.15-0007.

Cui, L. and Su, X. (2009) 'Discovery, mechanisms of action and combination therapy of artemisinin.', *Expert review of anti-infective therapy*. NIH Public Access, 7(8), pp. 999–1013. doi: 10.1586/eri.09.68.

Dahl, E. L. and Rosenthal, P. J. (2008) 'Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics', *Trends in Parasitology*. Elsevier Current Trends, 24(6), pp. 279–284. doi: 10.1016/J.PT.2008.03.007.

Davis, T. M. E. *et al.* (2005) 'Piperaquine', *Drugs*. Springer International Publishing, 65(1), pp. 75–87. doi: 10.2165/00003495-200565010-00004.

Dondorp, A. *et al.* (2005) 'Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial.', *Lancet (London, England)*. Elsevier, 366(9487), pp. 717–25. doi: 10.1016/S0140-6736(05)67176-0.

Dondorp, A. M. *et al.* (2009) 'Artemisinin Resistance in *Plasmodium falciparum* Malaria', *New England Journal of Medicine*, 361(5), pp. 455–467. doi: 10.1056/NEJMoa0808859.

Dondorp, A. M. *et al.* (2010) 'Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial.', *Lancet (London, England)*. Elsevier, 376(9753), pp. 1647–57. doi: 10.1016/S0140-6736(10)61924-1.

Dondorp, A. M. *et al.* (2017) 'How to Contain Artemisinin- and Multidrug-Resistant Falciparum Malaria.', *Trends in parasitology*. Elsevier, 33(5), pp. 353–363. doi: 10.1016/j.pt.2017.01.004.

VI-REFERENCES

Drugs, I. of M. (US) C. on the E. of A. *et al.* (2004) 'A Brief History of Malaria'. National Academies Press (US). Available at: https://www.ncbi.nlm.nih.gov/books/NBK215638/ (Accessed: 21 January 2019).

Duru, V. *et al.* (2015) 'Plasmodium falciparum dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations', *BMC Medicine*, 13(1), p. 305. doi: 10.1186/s12916-015-0539-5.

Famin, O. and Ginsburg, H. (2002) 'Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in Plasmodium falciparum-infected erythrocytes.', *Biochemical pharmacology*, 63(3), pp. 393–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11853690 (Accessed: 28 January 2019).

Fang, J. *et al.* (2013) 'Dihydro-orotate dehydrogenase is physically associated with the respiratory complex and its loss leads to mitochondrial dysfunction.', *Bioscience reports*. Portland Press Ltd, 33(2), p. e00021. doi: 10.1042/BSR20120097.

Farrar et al. (2014) *Manson ' s Tropical Diseases*. doi: 10.1016/B978-0-7020-5101-2.00090-X.

Ferone, R., Burchall, J. J. and Hitchings, G. H. (1969) 'Plasmodium berghei dihydrofolate reductase. Isolation, properties, and inhibition by antifolates.', *Molecular pharmacology*, 5(1), pp. 49–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4392112 (Accessed: 28 January 2019).

Fidock, D. A. *et al.* (2004) 'Antimalarial drug discovery: efficacy models for compound screening', *Nature Reviews Drug Discovery*. Nature Publishing Group, 3(6), pp. 509–520. doi: 10.1038/nrd1416.

Folarin, O. A. *et al.* (2011) 'In vitro amodiaquine resistance and its association with mutations in pfcrt and pfmdr1 genes of Plasmodium falciparum isolates from Nigeria.', *Acta tropica*. NIH Public Access, 120(3), pp. 224–30. doi: 10.1016/j.actatropica.2011.08.013.

Fu, Y. *et al.* (2010) 'Dual labeling with a far red probe permits analysis of growth and

oxidative stress in *P. falciparum* -infected erythrocytes', *Cytometry Part A*, 9999A, p. NA-NA. doi: 10.1002/cyto.a.20856.

Gunjan, S. *et al.* (2018) 'Artemisinin Derivatives and Synthetic Trioxane Trigger Apoptotic Cell Death in Asexual Stages of Plasmodium.', *Frontiers in cellular and infection microbiology*. Frontiers Media SA, 8, p. 256. doi: 10.3389/fcimb.2018.00256.

Haldar, K., Bhattacharjee, S. and Safeukui, I. (2018) 'Sporozoites Drug resistance in Plasmodium', *Nature Publishing Group*, 16. doi: 10.1038/nrmicro.2017.161.

Harris, C. B. *et al.* (2013) 'Dietary pyrroloquinoline quinone (PQQ) alters indicators of inflammation and mitochondrial-related metabolism in human subjects', *The Journal of Nutritional Biochemistry*. Elsevier, 24(12), pp. 2076–2084. doi: 10.1016/J.JNUTBIO.2013.07.008.

Hoffman, S. L. *et al.* (1985) 'RII and RIII type resistance of Plasmodium falciparum to combination of mefloquine and sulfadoxine/pyrimethamine in Indonesia.', *Lancet (London, England)*, 2(8463), pp. 1039–40. doi: 10.1016/S0140-6736(85)90908-0.

Hurwitz, E. S., Johnson, D. and Campbell, C. C. (1981) 'Resistance of Plasmodium falciparum malaria to sulfadoxine-pyrimethamine ('Fansidar') in a refugee camp in Thailand.', *Lancet (London, England)*, 1(8229), pp. 1068–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6112445 (Accessed: 25 January 2019).

Keravis, T. and Lugnier, C. (2012) 'Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments', *British Journal of Pharmacology*, 165(5), pp. 1288–1305. doi: 10.1111/j.1476-5381.2011.01729.x.

Knell, A. J. (1991) 'Malaria', *The Wellcome Thrust*, p. 97.

KRISHNA, S., UHLEMANN, A. and HAYNES, R. (2004) 'Artemisinins: mechanisms of action and potential for resistance', *Drug Resistance Updates*, 7(4–5), pp. 233–244. doi: 10.1016/j.drup.2004.07.001.

Krungkrai, S. R. and Krungkrai, J. (2016) 'Insights into the pyrimidine biosynthetic pathway of human malaria parasite Plasmodium falciparum as chemotherapeutic target',

Asian Pacific Journal of Tropical Medicine. No longer published by Elsevier, 9(6), pp. 525–534. doi: 10.1016/J.APJTM.2016.04.012.

Leang, R. *et al.* (2013) 'Efficacy of Dihydroartemisinin-Piperaquine for Treatment of Uncomplicated Plasmodium falciparum and Plasmodium vivax in Cambodia, 2008 to 2010', *Antimicrobial Agents and Chemotherapy*, 57(2), pp. 818–826. doi: 10.1128/AAC.00686-12.

Leang, R. *et al.* (2015) 'Evidence of Plasmodium falciparum Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment', *Antimicrobial Agents and Chemotherapy*, 59(8), pp. 4719–4726. doi: 10.1128/AAC.00835-15.

Lobo, L. *et al.* (2018) 'New endoperoxides highly active in vivo and in vitro against artemisinin-resistant Plasmodium falciparum', *Malaria Journal*. BioMed Central, 17(1), p. 145. doi: 10.1186/s12936-018-2281-x.

Lon, C. *et al.* (2014) 'Efficacy of Two versus Three-Day Regimens of Dihydroartemisinin-Piperaquine for Uncomplicated Malaria in Military Personnel in Northern Cambodia: An Open-Label Randomized Trial', *PLoS ONE*. Edited by L. von Seidlein. Public Library of Science, 9(3), p. e93138. doi: 10.1371/journal.pone.0093138.

Loria, P. *et al.* (1999) 'Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials.', *The Biochemical journal*. Portland Press Ltd, 339 (Pt 2)(Pt 2), pp. 363–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10191268 (Accessed: 28 January 2019).

Luth, M. R. *et al.* (2018) 'Using *in Vitro* Evolution and Whole Genome Analysis To Discover Next Generation Targets for Antimalarial Drug Discovery', *ACS Infectious Diseases*. American Chemical Society, 4(3), pp. 301–314. doi: 10.1021/acsinfecdis.7b00276.

Machado, M. *et al.* (2016) *Whole-Cell SYBR Green I Assay for Antimalarial Activity Assessment, Ann Clin Med Microbio*. Available at: https://run.unl.pt/bitstream/10362/36652/1/Whole_cell_SYBR_Green_I_assay_for_antimalarial_activity_assessment.pdf (Accessed: 29 July 2018).

VI-REFERENCES

Maerki, S. *et al.* (2006) 'In vitro assessment of the pharmacodynamic properties and the partitioning of OZ277/RBx-11160 in cultures of Plasmodium falciparum', *Journal of Antimicrobial Chemotherapy*. Oxford University Press, 58(1), pp. 52–58. doi: 10.1093/jac/dkl209.

Le Manach, C. *et al.* (2013) 'Fast in vitro methods to determine the speed of action and the stage-specificity of anti-malarials in Plasmodium falciparum', *Malaria Journal*. BioMed Central, 12(1), p. 424. doi: 10.1186/1475-2875-12-424.

Mohring, F. *et al.* (2017) 'Determination of glutathione redox potential and pH value in subcellular compartments of malaria parasites', *Free Radical Biology and Medicine*. Pergamon, 104, pp. 104–117. doi: 10.1016/J.FREERADBIOMED.2017.01.001.

Mohring, F., Jortzik, E. and Becker, K. (2016) 'Comparison of methods probing the intracellular redox milieu in Plasmodium falciparum', *Molecular and Biochemical Parasitology*. Elsevier, 206(1–2), pp. 75–83. doi: 10.1016/J.MOLBIOPARA.2015.11.002.

Monterisi, S. *et al.* (2017) 'PDE2A2 regulates mitochondria morphology and apoptotic cell death via local modulation of cAMP/PKA signalling.', *eLife*. eLife Sciences Publications, Ltd, 6. doi: 10.7554/eLife.21374.

Morrissette, N. S. and Sibley, L. D. (2002) 'Cytoskeleton of apicomplexan parasites.', *Microbiology and molecular biology reviews : MMBR*. American Society for Microbiology (ASM), 66(1), pp. 21–38; table of contents. doi: 10.1128/MMBR.66.1.21-38.2002.

Nixon, G. L. *et al.* (2013) 'Antimalarial pharmacology and therapeutics of atovaquone.', *The Journal of antimicrobial chemotherapy*. Oxford University Press, 68(5), pp. 977–85. doi: 10.1093/jac/dks504.

Noedl, H., Socheat, D. and Satimai, W. (2009) 'Artemisinin-Resistant Malaria in Asia', *New England Journal of Medicine*, 361(5), pp. 540–541. doi: 10.1056/NEJMc0900231.

Nogueira, F. and Rosário, V. E. do (2010) 'Methods for assessment of antimalarial activity in the different phases of the Plasmodium life cycle', *Revista Pan-Amazônica de*

Saúde, 1(3), pp. 109–124. doi: 10.5123/S2176-62232010000300015.

Nosten, F. and White, N. J. (2007) *Artemisinin-Based Combination Treatment of Falciparum Malaria*. Available at: http://www.ajtmh.org/docserver/fulltext/14761645/77/6_Suppl/0770181.pdf?expires=1548443022&id=id&accname=guest&checksum=AE488886A21CC949E0913A39505B76FA (Accessed: 25 January 2019).

Paguio, Michelle F, Bogle, K. L. and Roepe, P. D. (2011) 'Plasmodium falciparum resistance to cytocidal versus cytostatic effects of chloroquine.', *Molecular and biochemical parasitology*. NIH Public Access, 178(1–2), pp. 1–6. doi: 10.1016/j.molbiopara.2011.03.003.

Paguio, Michelle F., Bogle, K. L. and Roepe, P. D. (2011) 'Plasmodium falciparum resistance to cytocidal versus cytostatic effects of chloroquine', *Molecular and Biochemical Parasitology*. Elsevier, 178(1–2), pp. 1–6. doi: 10.1016/J.MOLBIOPARA.2011.03.003.

Petersen, I., Eastman, R. and Lanzer, M. (2011) 'Drug-resistant malaria: Molecular mechanisms and implications for public health', *FEBS Letters*. No longer published by Elsevier, 585(11), pp. 1551–1562. doi: 10.1016/J.FEBSLET.2011.04.042.

Phillips, M. A. and Rathod, P. K. (2010) 'Plasmodium dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy', *Infectious disorders drug targets*. NIH Public Access, 10(3), p. 226. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2883174/ (Accessed: 18 October 2018).

Ramdani, G. *et al.* (2015) 'cAMP-Signalling Regulates Gametocyte-Infected Erythrocyte Deformability Required for Malaria Parasite Transmission.', *PLoS pathogens*. Public Library of Science, 11(5), p. e1004815. doi: 10.1371/journal.ppat.1004815.

Raphemot, R. *et al.* (2016) 'Discovery of Dual-Stage Malaria Inhibitors with New Targets', *Antimicrobial Agents and Chemotherapy*, 60(3), pp. 1430–1437. doi: 10.1128/AAC.02110-15.

Roederer, M. (2001) 'Spectral compensation for flow cytometry: visualization artifacts,

limitations, and caveats.', *Cytometry*, 45(3), pp. 194–205. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11746088 (Accessed: 4 June 2019).

Roepe, P. D. (2014) 'To kill or not to kill, that is the question: cytocidal antimalarial drug resistance.', *Trends in parasitology*. NIH Public Access, 30(3), pp. 130–5. doi: 10.1016/j.pt.2014.01.004.

Roncalés, M. *et al.* (2015) 'In Vitro Culture of Plasmodium falciparum: Obtention of Synchronous Asexual Erythrocytic Stages', *Open Journal of Epidemiology*, 5, pp. 71–80. doi: 10.4236/ojepi.2015.51010.

Rosario, V. (1981) 'Cloning of naturally occurring mixed infections of malaria parasites.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 212(4498), pp. 1037–8. doi: 10.1126/SCIENCE.7015505.

Rosenthal, P. J. (2001) *Antimalarial chemotherapy : mechanisms of action, resistance, and new directions in drug discovery*. Humana Press.

Sanz, L. M. *et al.* (2012) 'P. falciparum In Vitro Killing Rates Allow to Discriminate between Different Antimalarial Mode-of-Action', *PLoS ONE*. Edited by T. Spielmann. Public Library of Science, 7(2), p. e30949. doi: 10.1371/journal.pone.0030949.

Schuck, D. C. *et al.* (2011) 'Flow cytometry as a tool for analyzing changes in Plasmodium falciparum cell cycle following treatment with indol compounds', *Cytometry Part A*. John Wiley & Sons, Ltd, 79A(11), pp. 959–964. doi: 10.1002/cyto.a.21136.

Sinha, S. *et al.* (2017) 'Development in Assay Methods for in Vitro Antimalarial Drug Efficacy Testing: A Systematic Review', *Frontiers in Pharmacology*, 8, p. 754. doi: 10.3389/fphar.2017.00754.

Spring, M. D. *et al.* (2015) 'Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study', *The Lancet Infectious Diseases*, 15(6), pp. 683–691. doi: 10.1016/S1473-3099(15)70049-6.

Srivastava, I. K., Rottenberg, H. and Vaidya, A. B. (1997) 'Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular

Biology, 272(7), pp. 3961–6. doi: 10.1074/JBC.272.7.3961.

Talman, A. M., Blagborough, A. M. and Sinden, R. E. (2010) 'A Plasmodium falciparum strain expressing GFP throughout the parasite's life-cycle.', *PloS one*. Public Library of Science, 5(2), p. e9156. doi: 10.1371/journal.pone.0009156.

Tizifa, T. A. *et al.* (2018) 'Prevention Efforts for Malaria', *Current Tropical Medicine Reports*. Springer International Publishing, 5(1), pp. 41–50. doi: 10.1007/s40475-018-0133-y.

Trenholme, C. *et al.* (1975) 'Mefloquine (WR 142,490) in the treatment of human malaria', *Science*, 190(4216), pp. 792–794. doi: 10.1126/science.1105787.

Verdrager, J. (1986) 'Epidemiology of emergence and spread of drug-resistant falciparum malaria in Southeast Asia.', *The Southeast Asian journal of tropical medicine and public health*, 17(1), pp. 111–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3526576 (Accessed: 25 January 2019).

Van Voorhis, W. C. *et al.* (2016) 'Open Source Drug Discovery with the Malaria Box Compound Collection for Neglected Diseases and Beyond', *PLOS Pathogens*. Edited by M. A. Phillips. Public Library of Science, 12(7), p. e1005763. doi: 10.1371/journal.ppat.1005763.

Walker, N. F., Nadjm, B. and Whitty, C. J. M. (2018) 'Malaria', *Medicine*. Elsevier, 46(1), pp. 52–58. doi: 10.1016/J.MPMED.2017.10.012.

Wein, S. *et al.* (2010) 'Reliability of antimalarial sensitivity tests depends on drug mechanisms of action.', *Journal of clinical microbiology*. American Society for Microbiology Journals, 48(5), pp. 1651–60. doi: 10.1128/JCM.02250-09.

Wernsdorfer, W. H. and Payne, D. (1991) 'The dynamics of drug resistance in Plasmodium falciparum.', *Pharmacology & therapeutics*, 50(1), pp. 95–121. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1891480 (Accessed: 25 January 2019).

White, L. J. *et al.* (2015) 'Defining the In Vivo Phenotype of Artemisinin-Resistant Falciparum Malaria: A Modelling Approach', *PLOS Medicine*. Edited by T. A. Smith, 12(4), p. e1001823. doi: 10.1371/journal.pmed.1001823.

White, N. J. *et al.* (2014) 'Malaria.', *Lancet (London, England)*. Elsevier, 383(9918), pp. 723–35. doi: 10.1016/S0140-6736(13)60024-0.

WHO (2010) *Global report on antimalarial druG efficacy and druG resistance*. Available at: https://apps.who.int/iris/bitstream/handle/10665/44449/9789241500470_eng.pdf?sequence=1 (Accessed: 5 June 2019).

WHO (2015a) *FOR THE TREATMENT OF MALARIA GUIDELINES*. Available at: www.who.int (Accessed: 4 October 2018).

WHO (2015b) *Reversing the Incidence of Malaria 2000-2015*. Available at: https://www.unicef.org/publications/files/Achieving_the_Malaria_MDG_Target.pdf (Accessed: 16 October 2018).

WHO (2017) *WORLD MALARIA REPORT 2017*. Available at: www.who.int/malaria (Accessed: 28 January 2019).

Wilson, D. W. *et al.* (2013) 'Defining the timing of action of antimalarial drugs against Plasmodium falciparum.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology Journals, 57(3), pp. 1455–67. doi: 10.1128/AAC.01881-12.

Yuthavong, Y. *et al.* (2006) 'Folate metabolism as a source of molecular targets for antimalarials', *Future Microbiology*, 1(1), pp. 113–125. doi: 10.2217/17460913.1.1.113.

Zarchin, S., Krugliak, M. and Ginsburg, H. (1986) 'Digestion of the host erythrocyte by malaria parasites is the primary target for quinoline-containing antimalarials.', *Biochemical pharmacology*, 35(14), pp. 2435–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3524576 (Accessed: 28 January 2019).

Zheng, H. *et al.* (2018) 'Design and synthesis of furyl/thineyl pyrroloquinolones based on natural alkaloid perlolyrine, lead to the discovery of potent and selective PDE5 inhibitors', *European Journal of Medicinal Chemistry*. Elsevier Masson, 150, pp. 30–38. doi: 10.1016/J.EJMECH.2018.02.039.

VII-ANNEXES

Table I: Ring-stage parasitaemia counted by microscope as opposed to parasitaemia got by cytometer and the respective percentage of efficacy of each compound at T48h and T96h.

Rings				
	Optical microscopy		**Flow cytometry**	
48h	**%Parasitaemia**	**% Efficacy of compounds**	**%Parasitaemia**	**% Efficacy of compounds**
K*	2,67	0,0	2,35	0,0
CQ	0,33	87,5	0,34	85,5
105	1,07	59,7	0,93	60,3
82	1,55	41,8	1,33	43,3
92	1,03	61,4	1,02	56,6
96h				
K*	6,80	0,0	3,99	0,0
CQ	0,06	99,2	0,04	99,0
105	0,26	96,1	0,21	94,7
82	1,90	72,1	1,41	64,7
92	0,57	91,7	0,35	91,3

*K: Untreated culture

Table II: Trophozoites-stage parasitaemia counted by microscope as opposed to parasitaemia got by cytometer and the respective percentage of efficacy of each compound at T48h and T96h.

Trophozoites				
	Optical microscopy		**Flow Cytometry**	
48h	**%Parasitaemia**	**% Efficacy of compounds**	**%Parasitaemia**	**% Efficacy of compounds**
K*	1,42	0,0	1,65	0,0
CQ	0,10	93,1	0,18	89,1
105	0,52	63,6	0,66	60,3
82	1,02	27,8	1,15	30,6
92	0,61	56,9	0,66	60,1
96h				
K*	1,57	0,0	1,37	0,0
CQ	0,05	96,8	0,03	98,1
105	0,21	86,4	0,23	83,4
82	1,43	9,0	1,25	8,2
92	0,38	76,1	0,32	76,8

*K: Untreated culture

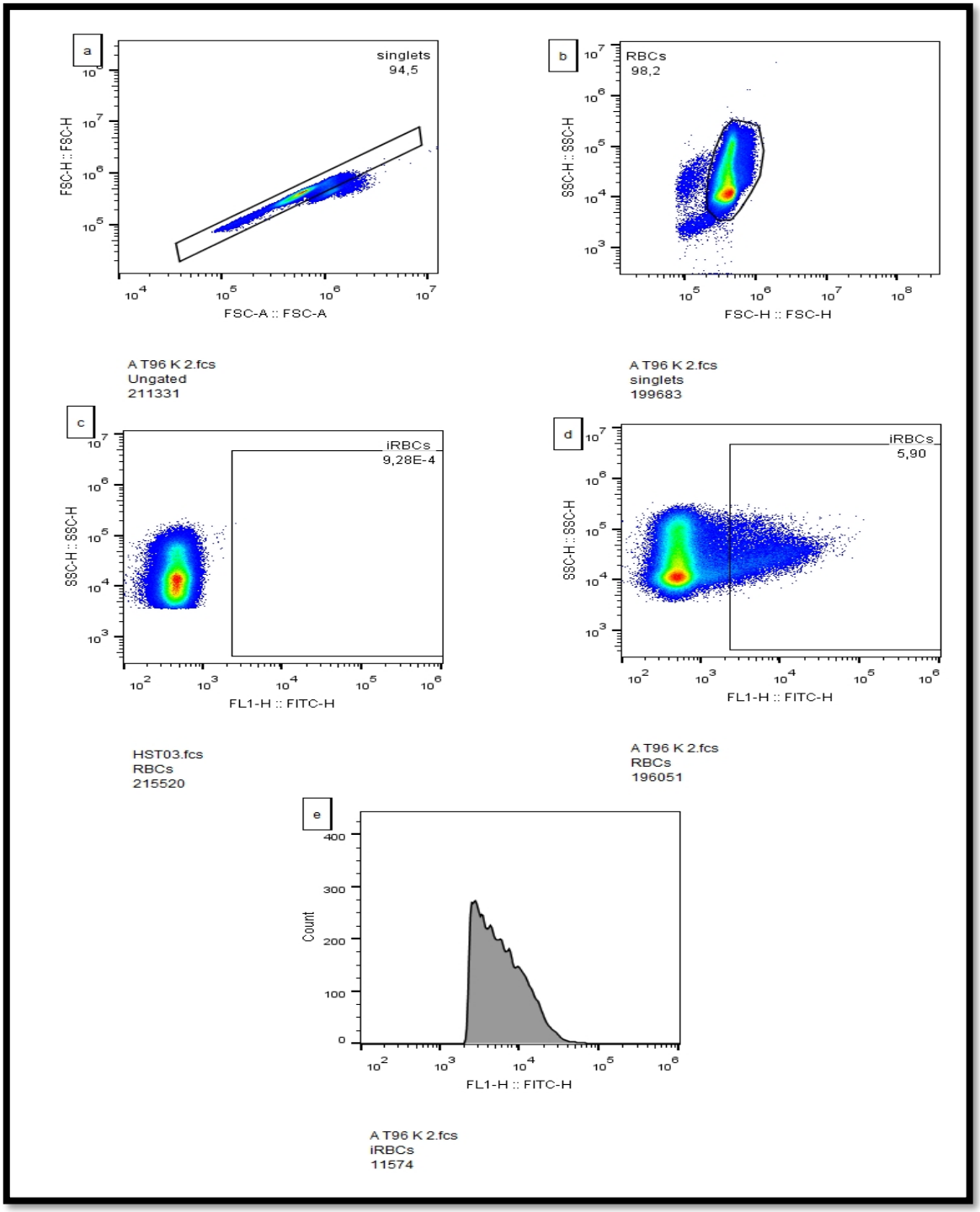

Figure I: Stage-Specific assay: flow cytometry analysis of ring-stage parasites.

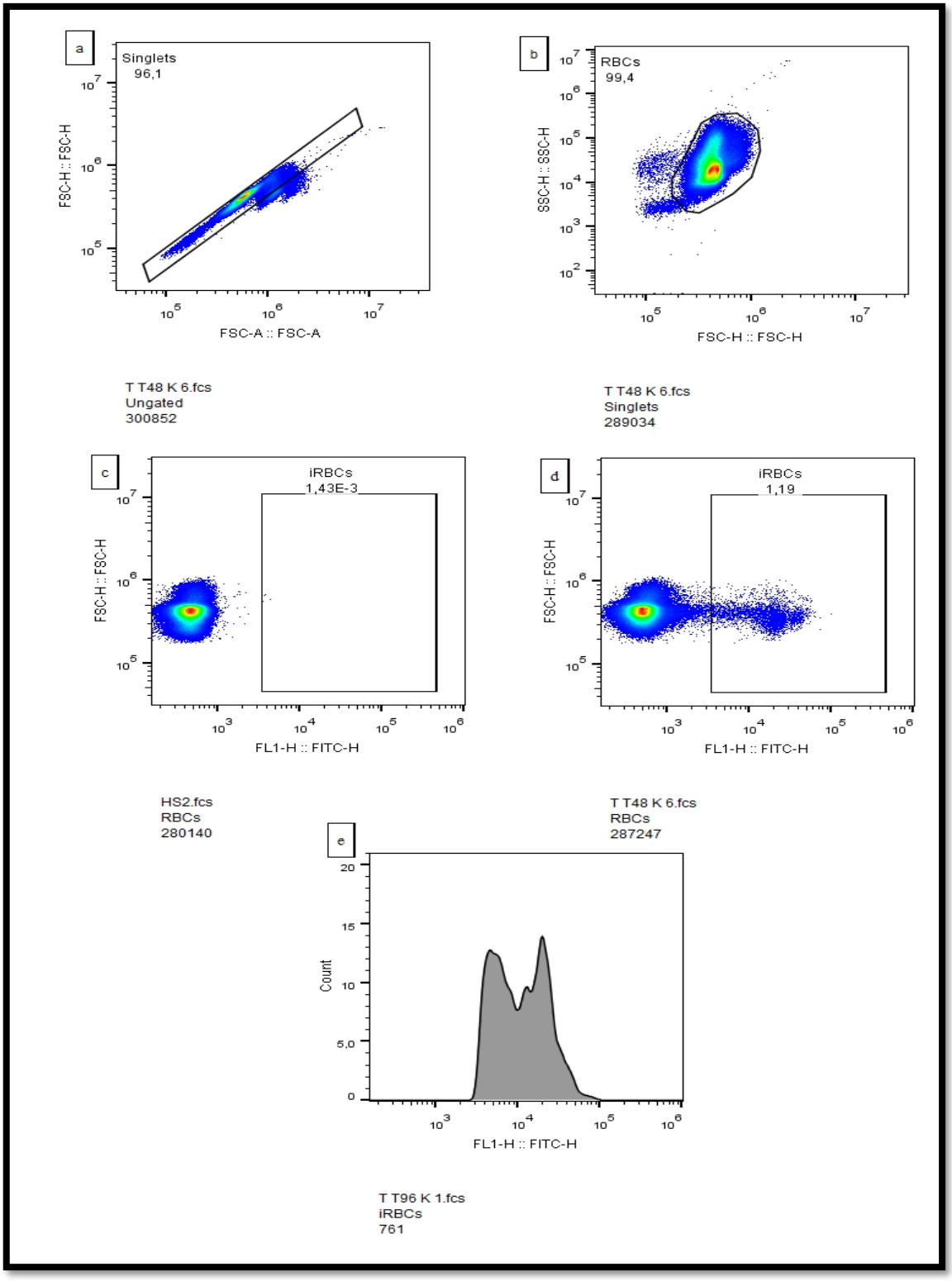

Figure II: Stage Specific assay: flow cytometry analysis of trophozoite-stages parasites.